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EXPLORING MTOR-DEPENDENT REGULATION OF MRNA TRANSLATION IN CANCER

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Exploring mTOR-dependent regulation of mRNA translation in cancer
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To my beloved family

ABSTRACT

Modulation of mRNA translation via the mammalian target of rapamycin (mTOR) pathway is primarily achieved by integrating internal or external signals onto the translation machinery, especially on the rate-limiting initiation step during mRNA translation. Subsets of transcripts are discriminated by structural and/or sequence features, and encode proteins involved in different biological functions. For example, a group of mRNAs with a stretch of uninterrupted 4-15 pyrimidines following a cytosine after the m^7Gppp cap at their 5' untranslated region (5'UTR) are termed as TOP mRNAs, which mainly encode ribosomal proteins and translation factors. By developing a luciferase reporter to quantify TOP mRNA translation, this thesis provides insight into mTOR-dependent or -independent modulators of TOP mRNA translation. These studies also suggest a vast kinase repertoire potentially modulating TOP-mRNA translation **(Paper II)**.

Aberrations in mTOR pathway drive tumorigenesis and development. The first-generation allosteric mTOR inhibitors, rapamycin analogs (also called rapalogs), and the second generation ATP-competitive kinase mTOR inhibitors have been tested in a wide range of tumors as monotherapy or a component of combination therapy. However, either the poor potency of the first generation or the toxicity of the second generation makes the clinical benefit limited. Based on the prototype of one third-generation mTOR inhibitor, RapaLink-1, we produced a series of new mTORC1-selective bi-steric inhibitors through continuous and finely tuned pharmaceutical and chemical modifications, which selectively inhibit mTORC1 over mTORC2, but retain potent efficacy in suppressing tumor growth **(Paper I)**. Findings within **Paper I** demonstrate that mTORC1 mediates modulation of mRNA translation.

Immune cells within the tumor microenvironment (TME) represent an indispensable factor during tumor cells' escape from immune surveillance, which occurs via multiple mechanisms, including immune cell acquisition of pro-tumor phenotypes. Therefore, immunotherapy, which re-activates the immune system towards cancer cells, has emerged as an essential treatment option. **Paper IV** indicates that immune suppression mediated by tumor-associated macrophage (TAM) depends on the MNK2/eIF4E axis but not mTOR. The model suggests that MNK2 thereby controls translation of a subset of transcripts encoding proteins which in turn modulate the TAM phenotype. This finding extends our understanding of how mRNA translation contributes to immune cell phenotypes.

Expression of a functional Von Hippel-Lindau Tumor Suppressor (VHL) protein is commonly lost in renal cell carcinoma (RCC). In RCC, there is ample prognosis heterogeneity among patients treated with rapalogs, which inhibits mTORC1. Thus, it could be due to acquired resistance. Therefore, We examined whether alterations in gene expression in response to rapalogs associated with *VHL* status **(Paper III)**. As expected, VHL re-expression not only caused wide-spread changes in mRNA levels but also alterations in mRNA translation. Moreover, translation of transcripts subsets was sensitive to rapamycin only under *VHL*

proficiency or *VHL* deficiency. Further studies will aim to determine whether these differences affect downstream phenotypes.

LIST OF SCIENTIFIC PAPERS

I. Selective Inhibitors of mTORC1 Activate 4EBP1 and Suppress Tumor Growth
Accepted by Nature Chemical Biology
Bianca J. Lee1*, Jacob A. Boyer*, G. Leslie Burnett, Arun P. Thottumkara, Nidhi Tibrewal, Stacy L. Wilson, Tientien Hsieh, Abby Marquez, Edward G. Lorenzana, James W. Evans, Laura Hulea, Gert Kiss, **Hui Liu**, Dong Lee, Ola Larsson, Shannon McLaughlan, Ivan Topisirovic, Zhengping Wang, Zhican Wang, Yongyuan Zhao, David Wildes, James B. Aggen, Mallika Singh, Adrian L. Gill, Jacqueline A. M. Smith#, Neal Rosen#

II. Identification of kinases modulating translation of mRNAs with terminal oligo-pyrimidine (TOP) motifs
Manuscript
Hui Liu, Vincent van Hoef, Johannes Ristau, and Ola Larsson

III. In vitro characterization of VHL dependent mRNA translation in renal cell carcinoma
Manuscript
Hui Liu*, Julia Vassalakis*, Kathleen Watt, Kristofferson Tandoc, Fernanda C S Lupinacci , Shannon McLaughlan, Martín Roffé, Ola Larsson#, Ivan Topisirovic#, Glaucia N.M. Hajj#

IV. MNK2 governs the macrophage antiinflammatory phenotype
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* Equal contributions

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CONTENTS

LIST OF ABBREVIATIONS

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AKT	protein kinase A
AMPK	AMP-activated protein kinase
ASO	antisense oligonucleotide
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
DEPTOR	DEP-domain-containing mTOR-interacting protein
DNA	deoxyribonucleic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
ER	endoplasmatic reticulum
eRF	eukaryotic release factors
ERK	extracellular signal-regulated kinase
FKBP12	FK506 binding protein 12
FRB	FKBP12-rapamycin binding domain
GCN2	general control non-derepressible 2
GDP	guanosine-di-phosphate
HIF	hypoxia-inducible factor
IGF-1	Insulin-like growth factor 1
IRES	internal ribosome entry site
ISR	integrated stress response
LARP1	La-related proein 1
MAPK:	mitogen-activated protein kinase
MCF-7:	Michigan Cancer Foundation-7
MEF	mouse embryonic fibroblast
MEK:	MAP (mitogen-activated protein) kinase/ERK (extracellular signal-regulated kinase)
Met-tRNAi	methionyl-initiatior transfer RNA
miRNA	microRNA
MNK	MAPK-interacting kinase

mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
p53	tumor protein p53
PABP	poly(A)-binding protein
PDCD4	programmed cell death protein 4
PERK	protein kinase R-like endoplasmatic reticulum kinase
PHD	prolyl hydroxylase domain protein
PI3K	phosphatidylinositol 3-phosphate kinase
PIC	pre-initiation complex
Raptor	regulatory-associated protein of mTOR
RBP	RNA binding protein
RCC	renal cell carcinoma
REDD1:	protein regulated in development and DNA damage response 1
Rheb	Ras homologue enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RNA	ribonucleic acid
Rnase	ribonuclease
RNAseq	RNA sequencing
rp	ribosomal protein
RPF	ribosome protected fragment
rpS6	ribosomal protein S6
rRNA	ribosomal RNA
RT-qPCR	reverse transcription quantitative polymerase chain reaction
S6K	p70 ribosomal S6 kinase
siRNA	silence RNA
TAM	tumor-associated macrophage
TC	ternary complex
TME	tumor microenvironment
TOP	terminal oligopyrimidine

tRNA	transfer RNA
TSC	tuberous sclerosis complex
TSS	transcription start site
uORF	upstream open reading frame
UPR	unfolded protein response
UTR	untranslated region
VEGF	vascular endothelial growth factor
VHL	Von Hippel-Lindau Tumor Suppressor

1 INTRODUCTION

1.1 GENE EXPRESSION

Gene expression is one of the most fundamental processes in eukaryotic cells. Genetic information stored in double-stranded DNA is transcribed into messenger RNA (mRNA) and mRNAs are then translated into proteins in a "DNA-RNA-protein" flow, which is referred to as the central dogma of molecular biology (Crick, 1970). Broadly speaking, regulation of gene expression drives cell proliferation, tissue differentiation, and organismal development. Regulation of gene expression controls timing, position, and amount of a given gene product. Narrowly speaking, it starts from transcription, RNA-splicing and -transport from the nucleus into the cytosol, to translation, mRNA decay and post-translational modification of a protein. For some proteins this is followed by transport to the cell surface or secretion. All these processes are dynamic and tightly controlled. As shown in **Figure 1**, multiple regulations at each step in the gene expression process will confer a specific layer (i.e. genome, transcriptome, proteome) of information as an output. Such control of gene expression plays an essential role in physiological and pathological conditions. Herein, messenger RNA (mRNA) translation will be the focus of this thesis.

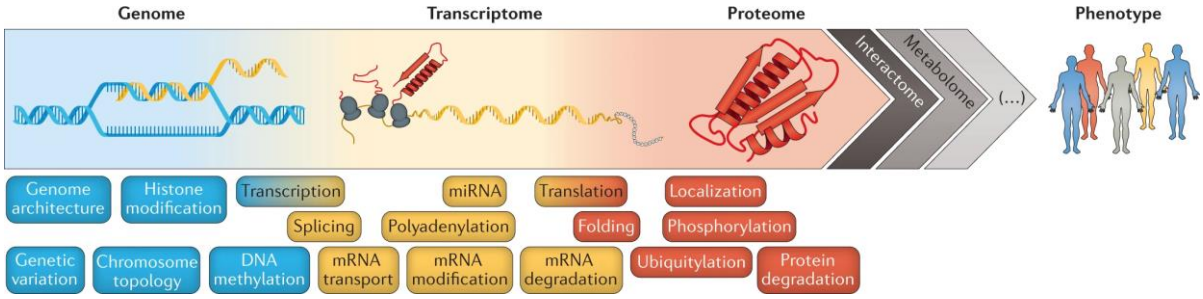

Figure 1: Overview of the gene expression pathway. Initial steps in gene expression connecting genotype to phenotype. Factors that confer control are indicated at the bottom.

Reprinted with permission from Springer Nature, Nature Reviews Genetics. Buccitelli, C., Selbach, M. mRNAs, proteins and the emerging principles of gene expression control. Nat Rev Genet 21, 630–644 (2020). https://doi.org/10.1038/s41576-020-0258-4.

1.2 TRANSLATION

Translation is a step-wise and coordinated process by which a polypeptide chain is synthesized from a messenger RNA (mRNA), during which ribosomes, transfer RNAs (tRNAs) and numerous other factors are engaged. It is one of the most energy-consuming steps as well as one of the most complex processes in cellular activity (Buttgereit & Brand, 1995). It can be divided into four phases: initiation, elongation, termination and ribosome recycling (Hershey, Sonenberg, & Mathews, 2012; Jackson, Hellen, & Pestova, 2010).

1.2.1 Translation initiation

Cap-dependent scanning mechanism for mRNA translation initiation is used by the majority of mRNAs in eukaryotes. The process of translation initiation includes the formation of several fundamental complexes, as well as the engagement of at least 12 proteins, i.e. eukaryotic initiation factors (eIFs) (**Figure 2**) (Jackson et al., 2010; Marintchev & Wagner, 2004; Merrick & Pavitt, 2018; Sonenberg & Hinnebusch, 2009; Thoreen, 2013). First, eIF2, initiator methionyl transfer RNA (Met-tRNA$_i$) and GTP form the ternary complex (TC), eIF2-GTP-Met-tRNA$_i$. This complex joins with the 40S small ribosomal subunit together with other eukaryotic initiation factors (eIFs; eIF1, eIF1A, eIF3, eIF5) to form the 43S ribosome pre-initiation complex (PIC). Secondly, the assembly of the cap-binding complex eIF4F happens at the m^{7}GpppN cap. It consists of eIF4E (cap-binding protein), eIF4G (scaffolding protein) and eIF4A (helicase). Finally, the 43S PIC is recruited to the 5' cap through interacting with eIF4G to form the 48S ribosome complex, where the 40S ribosomal subunit scans the mRNA from the 5'end towards the first start codon (typically AUG, or rarely a near-cognate AUG). During this process, eIF4G also interacts with the poly (A)-binding protein (PAPB), which stabilizes the mRNA and enhances translation via binding the poly (A) tail of the 3'UTR (Ivanov et al., 2016).

Once AUG recognition occurs, hydrolysis of eIF2-bound GTP is triggered and eIFs (including eIF2) are released from PIC. Then the large 60S ribosomal subunit joins to form the 80S ribosome, which will enter the translation elongation phase (Hinnebusch, 2014). As translation is a cyclic process, inactive eIF2-GDP will be converted to active eIF2-GTP by the guanine nucleotide exchange factor eIF2B, to participate in another round of initiation.

Besides the cap-dependent translation initiation mechanism in eukaryotes, translation initiation of some mRNAs is mediated by cap-independent mechanisms such as an internal ribosome entry site (IRES) (Hinnebusch, 2014; Jackson et al., 2010). IRESes, which are often in the 5'UTR, directly recruit ribosomes, bypassing the scanning of 5'UTR, and allow translation to proceed under physiological conditions when the cap-dependent translation is impaired.

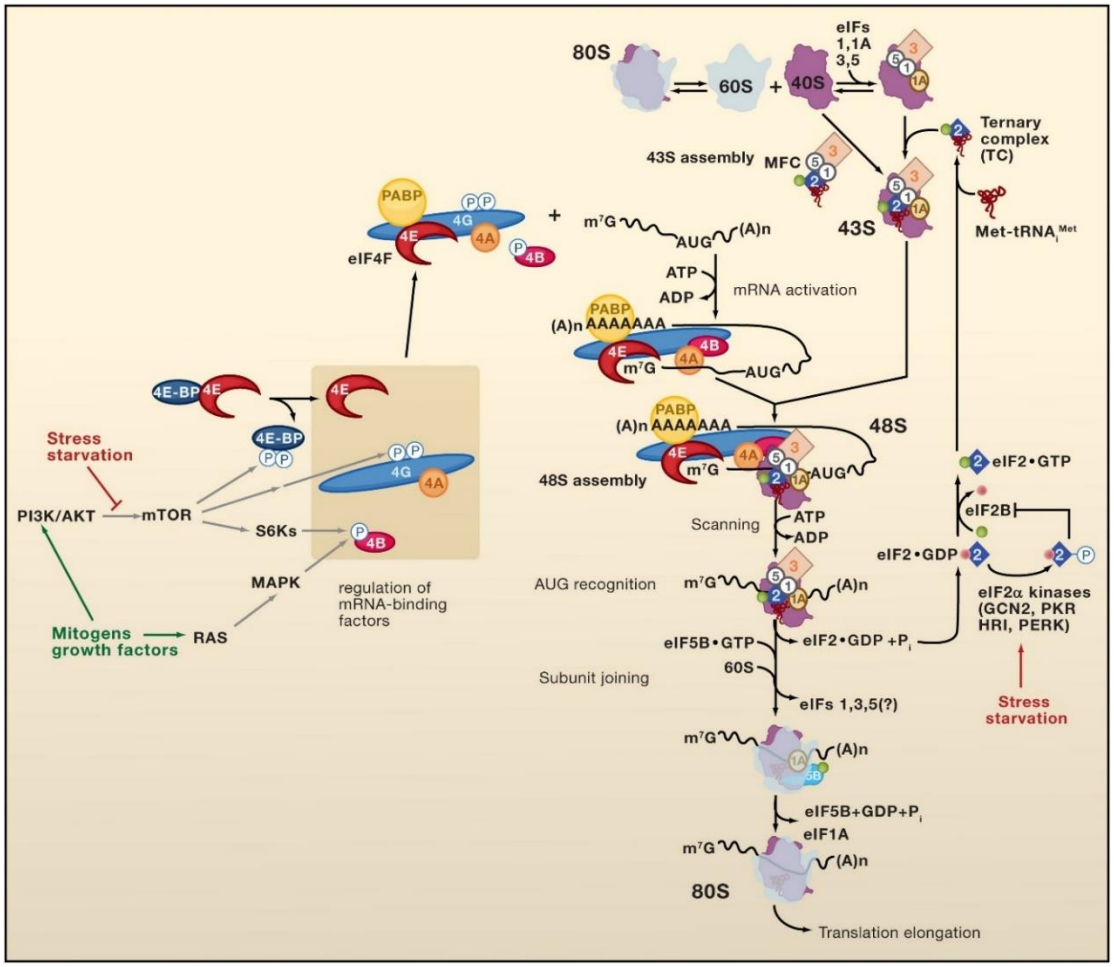

Figure 2: Cap-dependent translation initiation process in eukaryotes and regulatory factors of TC and eIF4F formation.

Reprinted with permission from Elsevier, Cell. Nahum Sonenberg, Alan G. Hinnebusch, Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets, Cell, Volume 136, Issue 4, 2009, Pages 731-745, ISSN 0092-8674, https://doi.org/10.1016/j.cell.2009.01.042.

1.2.2 Translation elongation

Mechanism of elongation is conserved between eukaryotes and bacteria, with more studies having been focusing on bacterial systems (Rodnina & Wintermeyer, 2009). Elongation starts upon the formation of 80S ribosome at the start codon, which is the start of an open reading frame (ORF). Anticodon of Met-tRNA$_i$ is base-paired with the start codon in the P site (for peptidyl) of the 80S ribosome, while the second codon is present in the A site (aminoacyl). The eukaryotic elongation factor eEF1A plays an important role in this phase, as it binds aminoacyl-tRNA and GTP forming a ternary complex, eEF1A-GTP-aminoacyl-tRNA. Thus, the anticodon of a cognate elongating tRNA will be base-paired with the second codon of the mRNA in the A site, triggering the hydrolysis of GTP by eEF1A. Released eEF1A-GDP is recycled to eEF1A-GTP by the exchange factor eEF1B and aminoacyl-tRNA is accommodated into the A site. Peptide bond formation is accompanied with the A- and P-site tRNAs shifting into hybrid states, resulting in the acceptor ends of the tRNAs moving to P and E (exit) sites, respectively (Behrmann et al., 2015; Budkevich et al., 2011; Moazed & Noller, 1989).

Translocation of the tRNAs to the E and P sites is promoted by the elongation factor eEF2. Then a deacylated tRNA occupies the E site and the peptidyl-tRNA is in the P site, leaving the A site vacant, allowing for the appropriate eEF1A-GTP-aminoacyl-tRNA binding (Dever, Dinman, & Green, 2018; Dever & Green, 2012).

1.2.3 Translation termination and ribosome recycling

Translation termination happens when a stop codon (UAA, UGA or UAG) enters the A site. Two release factors involved in this phase are eRF1 and eRF3 (Alkalaeva, Pisarev, Frolova, Kisselev, & Pestova, 2006; Dever & Green, 2012; Jackson, Hellen, & Pestova, 2012). Here, eRF1, eRF3 and GTP form an eRF1-eRF3-GTP ternary complex, where eRF1 is responsible for recognition of the stop codons and release of the nascent peptide from the P site peptidyl-tRNA, while eRF3 hydrolyzes the GTP and enhances peptide release. This ternary complex will be disassembled and its constituents are ready for recycling. Ribosome recycling is mediated by an important protein ABCE1, which splits the 80S ribosome to release the 60S subunit, followed by dissociation of deacylated tRNA and mRNA from the 40S subunit (Dever & Green, 2012; Hellen, 2018). Ribosomes, mRNAs and tRNA are recycled to participate in multiple rounds of translation.

1.3 TRANSLATIONAL CONTROL OF GENE EXPRESSION

1.3.1 Origins of translational control

Only a few years after the articulation of the central dogma of molecular biology (CRICK, 1958), the notion that protein synthesis can be altered via the efficiency of utilization of mRNAs to regulate gene expression emerged. In 1961, Jacob and Monod perceived and advanced the concept that in the elegant model for transcriptional control, the intermediate mRNA plays a key role in protein synthesis. (Jacob & Monod, 1961). Ten years later, Humphreys alluded to the “now classical conclusion” that in eggs, upon fertilization, silent mRNA is translationally activated (Humphreys, 1971). The earliest use of the term “translational control” traces back to 1968, by which date now well-known paradigms of translational control had all been established, e.g. developing embryos, reticulocytes, virus-and phage-infected cells, etc. The study of translational control increased in the 1960s when a large proportion of the translational apparatus was characterized. This signifies the pace in advancement of the understanding of protein synthesis (M. B. Mathews, Sonenberg, & Hershey, 2000).

Translational control is defined as a change in the rate (efficiency) of translation of one or more mRNAs, i.e., the number of completed protein products per mRNA and unit time (Hershey, Sonenberg, & Mathews, 2019; M. B. Mathews et al., 2000). Generally, during protein synthesis, the number of translation initiation events per unit time (approximately the number of bound ribosomes on one transcript) approximates the number of polypeptides, i.e., protein synthesis is determined to a large extent by the initiation rate. Moreover, compared with

elongation and termination phases, initiation has been shown to be the rate-limiting step during the process under most conditions (Lodish & Jacobsen, 1972; Tsung, Inouye, & Inouye, 1989; Walden, Godefroy-Colburn, & Thach, 1981).

Under certain circumstances, responses via altered transcription cause a delay in implementing corresponding changes at the protein level. However, adaption at the translation step can happen directly and rapidly. On the other hand, changes at the translational level are mostly reversible and fine-tuned, making it an economical and flexible mode in terms of energy cost.

1.3.2 Principles of general and selective control of translation

Although direct regulation of translation is not equally studied as transcription, it does play a crucial role under certain occasions such as nutrient deprivation and energy stress, and is emerging to be more studied in regulation of gene expression. Control of translation is mostly regulated at the initiation phase allowing rapid changes of cellular protein levels (translational output). Accordingly, a particular layer of output between transcriptome and proteome in **Figure 1**, emerges and is termed as **translatome**. Theoretically, regulation of eIFs activity and/or availability will affect translation of most mRNAs which use the cap-dependent scanning mechanism for initiation.

1.3.2.1 eIF2-GTP-Met-tRNA$_i$ ternary complex

One of the best-characterized examples for regulation of translation initiation is the formation of the eIF2-GTP-Met-tRNA$_i$ ternary complex, in which eIF2 can be phosphorylated by any of four known stress-sensing kinases (**Figure 2**). In response to a range of physiological changes or pathological stimuli, the integrated stress response (ISR) pathway is activated to attenuate global protein synthesis while simultaneously triggering a transcriptional program to restore cellular homeostasis in eukaryotes (Harding et al., 2003; Ron, 2002). The core event is phosphorylation of eIF2α, and the four homologous eIF2α kinases are PKR-like ER kinase (PERK, EIF2AK3), double-stranded RNA-dependent protein kinase (PKR, EIF2AK2), heme-regulated eIF2α kinase (HRI, EIF2AK1), and general control non-derepressible 2 (GCN2, EIF2AK4) (Donnelly, Gorman, Gupta, & Samali, 2013; Wek, Jiang, & Anthony, n.d.). For instance, the endoplasmic reticulum (ER) is the cellular compartment where folding proteins into proper structures occurs. Upon stress, incorrectly folded proteins will accumulate thus triggering the ER-stress response (Harding, Zhang, & Ron, 1999), like the unfolded protein response (UPR) (Gething & Sambrook, 1992) for relieving the ER stress. Consequently, kinases such as PERK phosphorylate eIF2α subunit on Ser51 (Harding et al., 1999; Vattem & Wek, 2004). Phosphorylated eIF2-GTP can still participate in translation initiation; however, after the release of phosphorylated eIF2-GDP, it will tightly bind and sequester eIF2B, abrogating its activity and resulting in the decrease of the available eIF2-GTP-Met-tRNAi ternary complex (M. Mathews, Sonenberg, & Hershey, 2007). Although the translation of most mRNAs is inhibited through this mechanism, certain mRNAs are stimulated at the translational level. For instance, activating transcription factor 4 (ATF4) expression level is increased

around 5-fold by PERK activation via an upstream open reading frame (uORF)-mediated mechanism (Vattem & Wek, 2004).

1.3.2.2 eIF4F complex

In cap-dependent translation initiation, ribosome recruitment is regulated mostly via the formation of eIF4F complex, which can be affected by phosphorylation of eIF4E-binding proteins (4E-BPs; mostly, 4E-BP1). 4E-BP1 is phosphorylated on multiple sites by mTORC1, then dissociates from eIF4E, promoting eIF4E interaction with eIF4G and the assembly of eIF4F complex. Thus, eIF4F is a node for integrating upstream signals on translation. In **paper I**, we compared effects of new mTORC1-selective bi-steric inhibitors on the translatome with one potent pan-mTOR inhibitor MLN0128. The results indicated the new generation of inhibitors showed high selectivity to mTORC1 and fewer side effects but retained potent targeting of substrates. On the other hand, eIF4E can be phosphorylated (on Ser209) by mitogen-activated protein kinase (MAPK)-interacting protein kinases (MNKs) 1 and 2 (Pyronnet et al., 1999; Waskiewicz et al., 1999), and phosphorylation of eIF4E is important during tumorigenesis (Wendel et al., 2007). Currently, it has been noted that although phosphorylation of eIF4E does not have a major impact on global translation, it does partially promote translation of a subset of mRNAs encoding proteins involved in tumor metastasis, e.g. SNAIL and MMP-3 (N. Robichaud et al., 2014). In **paper IV**, in a breast cancer mouse model, during tumor progression, tumor-associated macrophages (TAMs) are reprogrammed from a proinflammatory phenotype towards an antiinflammatory phenotype with augmented phosphorylation of eIF4E via a selective translational program depending on MNK2 signaling (Bartish et al., 2020). Besides, phosphorylation of other factors such as eIF4G and ribosomal protein S6 (rpS6) (rpS6 is activated by S6K, downstream of mTORC1 pathway) has also been studied to mediate control of translation (Pende et al., 2004; Raught et al., 2000; Ruvinsky et al., 2005).

1.3.2.3 RNA-binding proteins (RBPs) and microRNAs (miRNAs)

RNA-binding proteins (RBPs) and microRNAs (miRNAs) are another two categories of factors regulating translation. Early studies found that most regulation by RBPs and microRNAs is inhibitory (De Melo Neto, Standart, & De Sa, 1995). Currently, more and more RBPs and microRNAs are recognized to stimulate translation, while some have dual regulation depending on contexts, e.g. La-related protein 1 (LARP1) suppresses translation via binding 5'-end of TOP mRNA with DM15 (DM15 is the C-terminal region of LARP1) (Fonseca et al., 2015a; Lahr et al., n.d.; Philippe, Vasseur, Debart, & Thoreen, 2018), while its La module (LaMod) domain remains constitutively bound to poly A tail-binding protein (PABP) to stabilize TOP mRNA, and whereby to facilitate translation (J.-J. Jia et al., 2021a). Due to the natural sequence-binding feature for their role in such control, RBPs and miRNAs have the potential to selectively regulate certain mRNAs with specific sequence features.

1.3.2.4 The TOP-motif enables mTOR-dependent regulation of mRNA translation

Ribosomal proteins and translation factors are encoded by mRNAs with a stretch of pyrimidines following a cytosine after the m^7Gppp cap [termed as 5' terminal oligopyrimidine (TOP motif); the corresponding mRNA is termed TOP mRNA] (Hamilton, Stoneley, Spriggs, & Bushell, 2006). This feature is necessary and must be in its native position for control of TOP mRNA translation (Meyuhas & Kahan, 2015). A series of studies have characterized their poor translation in quiescent cells but strong and rapid activation on nutrients refueling such as serum, amino acids or by insulin and have pinpointed the mTOR pathway in control of TOP mRNA translation (**Figure 3**) (Hsieh et al., 2012; Thoreen et al., 2012). However, the detailed molecular mechanism remains incompletely revealed. Most recently, La-related protein 1 (LARP1) was proposed for its negative role downstream of mTORC1 signaling to suppress TOP mRNA translation, although deletion of it could not completely de-repress the inhibition of rapamycin or torin1 on TOP mRNA translation (Aoki et al., 2013; Fonseca et al., 2015a, 2015b; J.-J. Jia et al., 2021b; Lahr et al., n.d.). Thus, we hypothesized that additional regulators exist whose effect on TOP mRNA translation may depend on mTORC1/2 or not. In **Paper II**, we generated a cell line stably expressing luciferase with the RPL23 TOP-motif which quantifies TOP mRNA translation. Further, we used this model in a high-throughput screening based on a kinome-wide siRNA library, aiming to explore potential targets positively or negatively regulating TOP mRNA translation. More details are provided in section 3.1.

1.3.2.5 mRNAs with long and structured 5'UTR

Because the mechanism of translation initiation for most mRNAs is cap-dependent, it is natural that mRNAs with long and structured 5'UTR are demanding regarding the availability of eIF4F complex (Svitkin et al., 2001) due to the need of eIF4A helicase activity (**Figure 3**). Specifically, mRNAs encoding proteins involved in cell growth, proliferation (e.g. cyclins, *ODC*, *VEGF* and *MYC*) are among those sensitive to eIF4E (Bhat et al., 2015a; Chu & Pelletier, 2018; Gandin et al., 2016; Nandagopal & Roux, 2015; Rousseau, Kaspar, Rosenwald, Gehrke, & Sonenberg, 1996; Sonenberg & Gingras, 1998; Thoreen, 2013). Consistent with this, overexpressed eIF4E has been associated with tumorigenesis and tumor progression (Y. Jia, Polunovsky, Bitterman, & Wagner, 2012). Recently, a subset of mRNAs involved in mitochondrial function and biogenesis are also emerging to be sensitive to eIF4E, but without long and structured 5'UTR, implying mechanism of the eIF4E sensitivity yet to be resolved (Gandin et al., 2016; Morita et al., 2013).

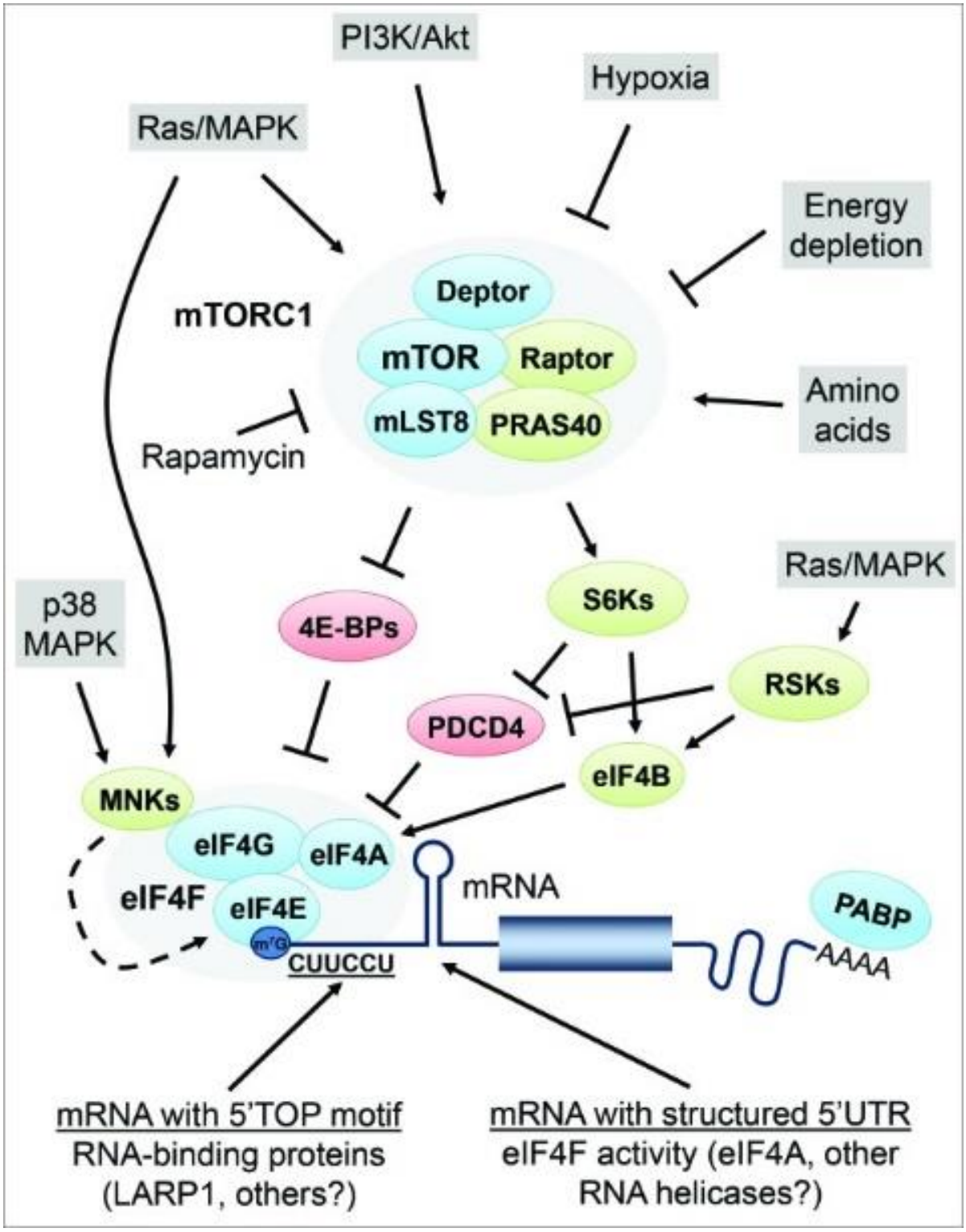

Figure 3: mTORC1 converges signaling to the translation machinery. Two groups of mRNAs are sensitive to mTORC1-mediated regulation: mRNA with TOP motif; mRNA with structured 5'UTR.

Reprinted with permission from Taylor & Francis, translation. Nandagopal N, Roux PP. Regulation of global and specific mRNA translation by the mTOR signaling pathway. Translation (Austin). 2015;3(1):e983402. Published 2015 Feb 2. doi:10.4161/21690731.2014.983402.

1.3.3 Translational control under different contexts

As mentioned above, multiple external or internal stimuli converge influences on several key components of translational apparatus, such as eIF4E and eIF2α. During physiological development, cells can be subjected to different stimuli, including hormones, growth factors, oxygen levels, nutrient deprivation, etc. Under certain conditions, translational control plays a more prominent role in maintaining homeostasis.

1.3.3.1 Nutrients and hormones converge signals via the mTOR pathway

1.3.3.1.1 mTOR

Studies leading to the discovery of mTOR started in the late 1970s with the discovery of rapamycin, which was first isolated from the *bacterium Streptomyces hygroscopicus* (Sehgal, Baker, & Vézina, 1975; Vézina & Kudelski, 1975). Functionally, rapamycin was found to suppress fungi growth, immune response and tumor growth (Eng, Sehgal, & Vézina, 1984; Sehgal et al., 1975). Afterwards, the two identified targeted proteins (encoded by *TOR1* and *TOR2*) were named the mechanistic target of rapamycin (mTOR) (Heitman, Movva, & Hall, 1991). From this point, mTOR has been widely studied and emerged to be a central node to converge multiple upstream signaling pathways on cellular anabolic and catabolic activities (Liu & Sabatini, 2020). As such, it plays an essential role in sensing nutrient availability to maintain cellular homeostasis.

mTOR is a serine/threonine protein kinase, mainly existing in two complexes named mTORC1 and mTORC2. These two complexes are distinguished by distinct subunits: Raptor (for mTORC1) and Rictor (for mTORC2), as well as by their unique substrates and sensitivity to rapamycin (Wullschleger, Loewith, & Hall, 2006). mTORC1 comprises three core components: mTOR, mammalian lethal with SEC13 protein 8 (mLST8, also known as GβL) (D. H. Kim et al., 2003) and regulatory-associated protein of mTOR (RAPTOR) (D.-H. Kim et al., 2002) (**Figure 4**). RAPTOR works as a scaffolding protein for mTORC1 accessory factor proline-rich AKT substrate 40KDa (PRAS40) (Sancak et al., 2007), which is an endogenous inhibitor of mTORC1, alongside DEP-domain-containing mTOR-interacting protein (DEPTOR) (Peterson et al., 2009).

Structural studies have reported the key residues in the kinase domain of mTOR may only shift into a catalytic position after binding the small GTPase Rheb which acts as an essential activator of mTOR (Yang et al., 2017). It has also revealed that the basis of mTORC1 inhibition by FKBP12, which binds the FKBP12-rapamycin binding (FRB) domain of mTOR, thus occluding the binding of substrate with the kinase active site (Yang et al., 2017, 2013). By contrast, in mTORC2, in lieu of RAPTOR, the scaffolding protein, RICTOR blocks the FRB domain on mTOR (**Figure 4**), rendering mTORC2 insensitivity to rapamycin. This provides a basis for selective targeting approaches. However, it has been noted prolonged rapamycin exposure will reduce assembly of mTORC2 via FKBP12-rapamycin binding to free mTOR molecule (Sarbassov et al., 2006).

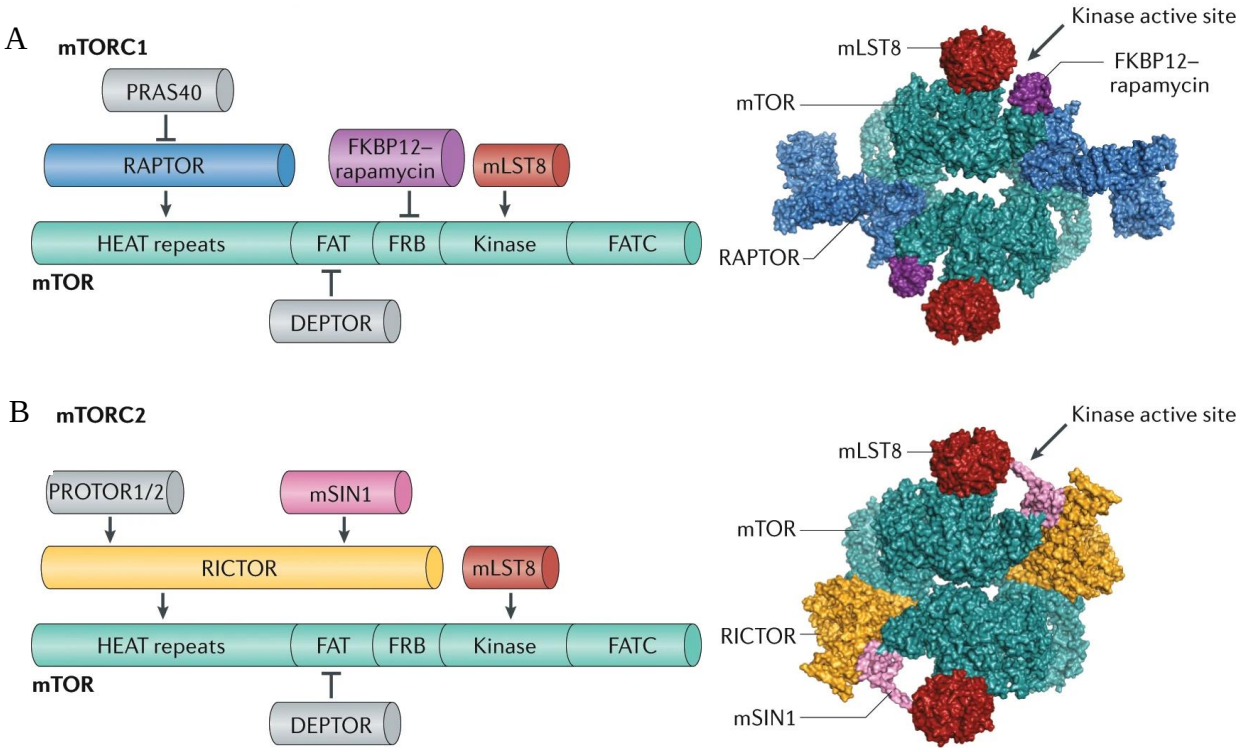

Figure 4: Structure of mTORC1(A) and mTORC2(B).

Modified and Reprinted with permission from Springer Nature, Nature Reviews Molecular Cell Biology. Liu, G.Y., Sabatini, D.M. mTOR at the nexus of nutrition, growth, ageing and disease. Nat Rev Mol Cell Biol 21, 183–203 (2020). https://doi.org/10.1038/s41580-019-0199-y.

As protein synthesis is the most energy-consuming and resource-intensive process, especially for fast-growing cells (Buttgereit & Brand, 1995), it is tightly regulated by mTORC1, mainly via the two substrates 4E-BP1 and p70 S6 kinase 1 (S6K1). The role of phosphorylation of 4E-BP1 has been described above. mTORC1 phosphorylates S6K1 on T389 (Burnett, Barrow, Cohen, Snyder, & Sabatini, 1998), which subsequently phosphorylates ribosomal protein S6 (rpS6), a component of the small 40S subunit of ribosome. Though some evidence suggests that rpS6 phosphorylation promotes ribosome biogenesis via a transcriptional program (Chauvin et al., 2014), the function of rpS6 phosphorylation remains ambiguous. Notably, rapamycin preferentially inhibits S6K1 durably, where 4E-BP1 recovers its phosphorylation within 6 hours (Choo, Yoon, Sang, Roux, & Blenis, 2008). In **paper I**, more details about selective targeting while remaining strong potency for mTORC1 in a pre-clinical experiment to treat cancer were provided.

1.3.3.1.2 Growth factors

Growth factors and other mitogens can converge their effects on the tuberous sclerosis complex (TSC1/TSC2), which is a GTPase-activating protein (GAP) for conversion of active Rheb-GTP state to the inactive Rheb-GDP state (Inoki, Li, Xu, & Guan, 2003). Through this, TSCs act as essential upstream regulators of mTORC1 activation (Valvezan & Manning, 2019). For example, insulin/insulin-like growth factor 1 (IGF-1) can activate Akt, whereby TSC2 gets phosphorylated and dissociates from the lysosomal surface and relieve inhibition of Rheb and mTORC1.(Demetriades, Doumpas, & Teleman, 2014; Garami et al., 2003). Apart from TSCs,

PRAS40 as mentioned above is an endogenous inhibitor of mTORC1, which associates with the scaffolding protein RAPTOR to abolish Rheb-driven mTORC1 activation (Haar, Lee, Bandhakavi, Griffin, & Kim, 2007; Sancak et al., 2007). PRAS40 can be phosphorylated not only by mTORC1 but also by Akt to relieve the inhibitory constraint of mTORC1 (Wiza, Nascimento, & Ouwens, 2012). How this coordination occurs remains to be elucidated.

1.3.3.1.3 Amino acids

Amino acids are among the major nutrients, and as early as in 1998, it was reported that leucine and arginine are required for mTORC1 activity (Hara et al., 1998). However, it is not until 2008 that the identification of Rag proteins via binding RAPTOR revealed the mechanism of mTORC1 in response to amino acid availability (E. Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008; Sancak et al., 2008). Specifically, Rags are a family of four related small guanosine triphosphatases (GTPases) (RagA, RagB, RagC, and RagD) and configured to be heterodimers, i.e. RagA or RagB is bound to RagC or RagD. They exist in two conformations being anchored to the lysosome by a pentameric Ragulator complex (comprising p18, p14, MP1, C7orf59 and HBXIP) (Bar-Peled, Schweitzer, Zoncu, & Sabatini, 2012; Sancak et al., 2010; Su et al., 2017): RagA/B-GTP coupled with RagC/D-GDP is in an active state; RagA/B-GDP coupled with RagC/D-GTP is inactive (Bar-Peled et al., 2012; Sancak et al., 2010; Shen, Choe, & Sabatini, 2017; Su et al., 2017). Rags, via interaction with RAPTOR, mediates the recruitment of mTORC1 from the cytosol to the lysosome, where Rheb stimulates the mTORC1 activity (Rogala et al., 2019). Currently, the study of amino acid sensors are still in exploration (Chantranupong et al., 2014; Saxton, Chantranupong, Knockenhauer, Schwartz, & Sabatini, 2016; Saxton, Knockenhauer, et al., 2016; Wolfson et al., 2016; Ye et al., 2015a).

GCN2 is highly conserved from yeast to humans (Castilho et al., 2014), while mammalian GCN2 is less studied (Caballero-Molada et al., 2018; Li et al., 2018b; Ye et al., 2015b). Under amino acid deficiency, GCN2 is activated by the binding of increased levels of deacylated tRNAs (Lageix, Zhang, Rothenburg, & Hinnebusch, 2015). As outlined in section 1.3.2.1, GCN2 is one of four stress-sensing kinases that respond to ISR and phosphorylates eIF2α to reduce global protein synthesis, simultaneously inducing a transcriptional program via ATF4's expression (Pakos‐Zebrucka et al., 2016). Sestrin2, a member of the Sestrin family of stress response proteins, which are emerging to be negative regulators of the Rag GTPases (Chantranupong et al., 2014; J. S. Kim et al., 2015; Parmigiani et al., 2014; Peng, Yin, & Li, 2014), is pinpointed to be induced via GCN2-ATF4 pathway under amino acid starvation (Ye et al., 2015b). Combing with the essential role of Rag GTPases in recruiting mTORC1 to the lysosome, sestrin2 whereby disrupts mTORC1 localization to the lysosome and sustains mTORC1 suppression (Ye et al., 2015b). Nonetheless, how GCN2 activation and mTORC1 inhibition are coordinated to regulate protein synthesis is still not clear.

Collectively, nutrients and hormonal signals are converging on translation machinery via mTORC1 and upstream pathways (**Figure 4, 5**).

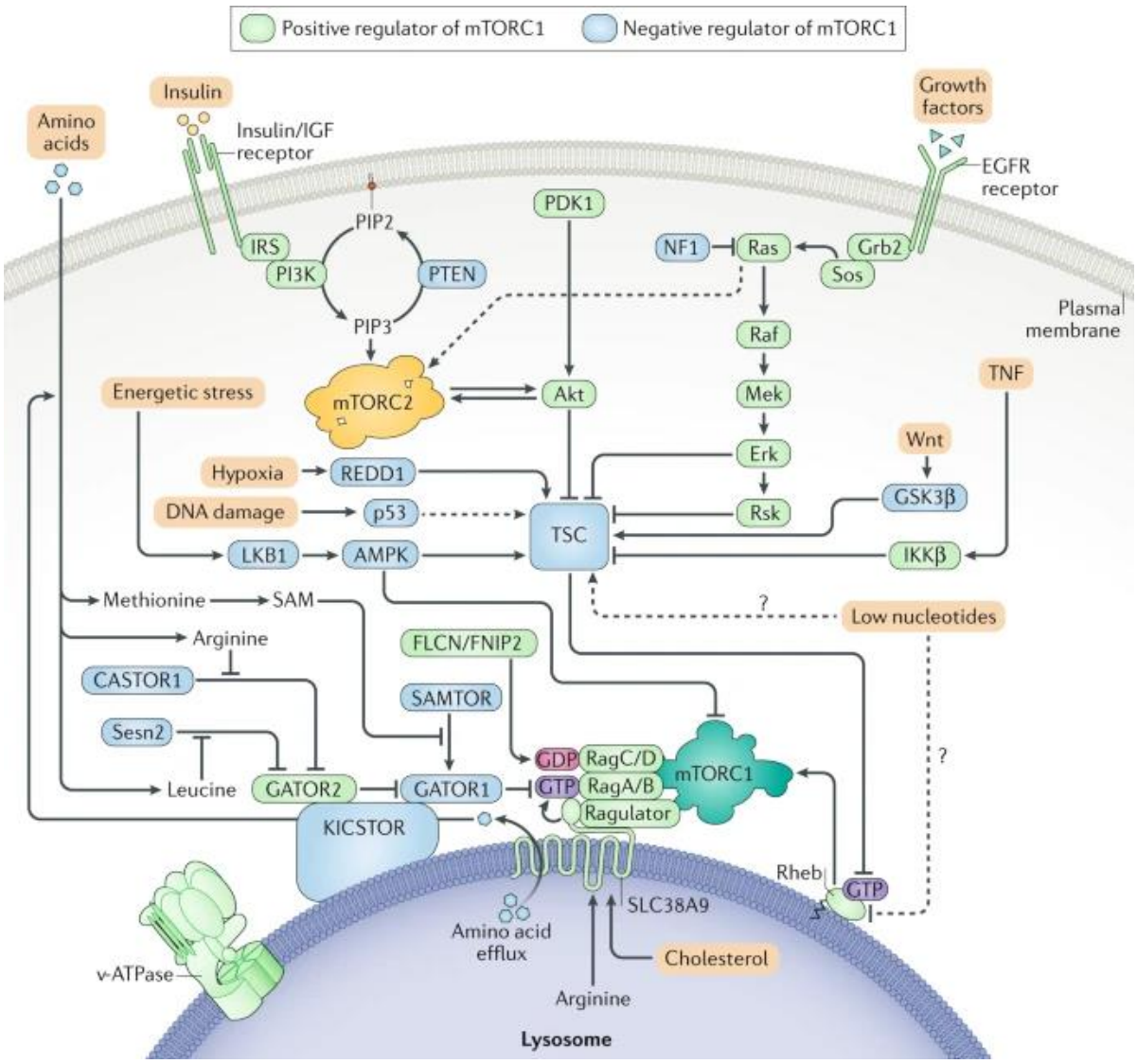

Figure 5: Regulatory network of the mTOR signaling pathway.

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1.3.3.2 Oxygen level, and VHL loss of function impinging on mRNA translation

For sensing oxygen levels, which is essential for cell growth and metabolism, prolyl hydroxylase domain protein (PHD) plays a critical role. PHD regulates hydroxylation of hypoxia-inducible factor α (HIFα), which is a transcription factor responsible for induction of genes involved in oxygen-independent glycolysis and angiogenesis for adaption of low oxygen condition (Gordan, Thompson, & Simon, 2007; Kaelin & Ratcliffe, 2008). PHD, as a member of dioxygenase family, uses molecular oxygen and α-ketoglutarate (α-KG) to hydroxylate the conserved prolyl residues in HIFα. Thereby, hydroxylated HIFα can be recognized by pVHL, which acts as a substrate recognition subunit of an E3 ubiquitin ligase. Recognized hydroxylated HIFα is ubiquitinated by the pVHL-E3 ubiquitin ligase and consequently gets cleared up by the proteasome system. Under normoxic condition, HIFα hydroxylation is dependent on the activity of PHD, resulting in rapid hydroxylation and degradation to maintain

a low HIFα level. Instead upon low oxygen, referred to as hypoxia, PHD activity is decreased due to the insufficient oxygen level. Therefore, HIFα is less hydroxylated and less degraded. Accumulated HIFα promotes gene expression necessary for adaption to this condition (Gordan et al., 2007; Majmundar, Wong, & Simon, 2010).

Current knowledge of translational control in response to hypoxia has emphasized a rapid inhibition of protein synthesis at the acute phase and maintained repression at the chronic phase via eIF2α and eIF4F complex, respectively (Koritzinsky et al., 2006; Koumenis et al., 2002). The availability of eIF2-GTP-Met-tRNA$_i$ ternary complex is a key point for regulation of translation, as described in sections 1.2.1 & 1.3.2.1. Under hypoxia, phosphorylation of eIF2 occurs within 1 hour due to a coordinated ER stress-activated PERK activation, which leads to inhibition of protein synthesis without any significant changes in transcription (Koumenis et al., 2002). Remarkably, phosphorylation of eIF2 reaches a maximum at 1-2 hours, and then declines regardless of the global translation inhibition. Furthermore, the phosphorylation is completely reversible upon re-oxygenation (Koumenis et al., 2002).

Inhibition of mRNA translation during prolonged hypoxic exposure is mediated by eIF4F, which is an eIF2α-independent mechanism. It has been shown that HIFα also induces Regulated in DNA damage and development 1 protein (REDD1) expression (also known as RTP801 or DDIT4) which restores TSC2 activity, and thereby suppresses mTORC1 activity to regulate protein synthesis (Brugarolas et al., 2004). Thus, the second mediator in control of translation during hypoxia, eIF4F, is at least partially controlled by HIFα-REDD1-TSC1/2 axis-dependent mTORC1 activity. There is also evidence that hypoxia inhibits mTORC1 partially through AMP-activated protein kinase (AMPK) (Corradetti, Inoki, & Guan, 2005; Sofer, Lei, Johannessen, & Ellisen, 2005; Wouters et al., 2005). Seemingly, it remains unclear how the multifaceted modulators coordinate in response to prolonged hypoxia (Van Den Beucken, Koritzinsky, & Wouters, 2006).

One specific context is *VHL* loss of function which is the most common genetic alteration in renal tumors, especially the most common histological type, clear-cell renal cell carcinoma (ccRCC). Inactivated *VHL* leads to constitutive HIF1α activation (Maxwell et al., 1999; Talks et al., 2000; Zhong et al., 1999), which may be expected to induce REDD1 and suppress mTORC1 activity. On the other hand, mTOR pathway is frequently hyperactivated in cases of ccRCC. Although the insensitivity and acquired resistance to rapalogs occurred in clinic, there was a study that reported observation of RCC with mutations in mTOR or related pathways benefiting from rapalogs (Kwiatkowski et al., 2016). In between of VHL and mTORC1, HIF may affect hyperactivated mTOR pathway or normal mTOR pathway in ccRCC differently depending on the status of *VHL*. Combining with the heterogeneous benefits of rapalogs in clinic and the lack of biomarkers for selection of RCC patients to rapalogs, it is necessary to look into the molecular mechanisms of interplay between VHL status and mTOR inhibition. In **paper III**, we specifically explored the VHL-associated translational control in response to one typical mTOR inhibitor, rapamycin using an in vitro renal cell carcinoma cell model.

1.4 DYSREGULATED TRANSLATION IN CANCER AND ITS THERAPEUTIC TARGETING

Early studies noted there were changes in the amount of unique mRNA species associated with polysomes when comparing resting to growing cells (Williams & Penman, 1975), i.e. some mRNAs in resting cells were not associated to polysomes in growing cell and, vice versa. Nowadays, dysregulated translation in cancer has been extensively reviewed (Nathaniel Robichaud, Sonenberg, Ruggero, & Schneider, 2019; D. Ruggero, 2012; Silvera, Formenti, & Schneider, 2010). As discussed in section 1.3.2.2, aberrant eIF4F function is implicated in a range of tumor types (C. N. Chen, Hsieh, Cheng, Lee, & Chang, 2004; Coleman et al., 2009; Graff et al., 2009; Holm et al., 2008; Rosenwald et al., 1999; Salehi, Mashayekhi, & Shahosseini, 2007; R. Wang et al., 2009; S. Wang et al., 1999). Among all translation factors, overexpression of eIF4E has been shown to promote tumorigenesis and metastasis (Davide Ruggero et al., 2004; Wendel et al., 2004), while overexpression of 4E-BP1 (a suppressor of eIF4E) partially reverses tumorigenesis(Rousseau, Gingras, Pause, & Sonenberg, 1996).

Several pathways, e.g. PI3K/mTOR, MEK-ERK, p38 MAPK impinge on the translation machinery, commonly on eIF4E, and thus affect translational output (Roux & Topisirovic, 2012, 2018). Accordingly, targeting mRNA translation via blocking upstream signaling pathways or direct interference with components of translation machinery is an emerging and interesting option for future cancer treatment (**Figure 6, Table 1**).

1.4.1 Targeting the mTOR pathway

Dysregulation of the mTOR pathway has been linked to many human diseases, including cancer (Bhat, Sonenberg, & Gores, 2013; Guertin & Sabatini, 2007; Laplante & Sabatini, 2012). As discussed in section 1.3.3.1, mTORC1 integrates a number of upstream signals onto the translation machinery. For instance, 4E-BP1, which is one direct substrate of mTORC1, controls the assembly of the eIF4F complex via binding eIF4E. Moreover, anti-tumor potency by suppressing this pathway is mainly achieved through hindering mTORC1 functionality (Janes & Fruman, 2010; Thoreen et al., 2009). During the past decades, mTOR inhibitors have been extensively developed. The first-generation inhibitors, rapamycin analogues, selectively inhibit mTORC1 via allosteric binding the FRB domain of mTOR in complex FKB12-rapamycin (**Figure 4**). Two rapalogs, everolimus and temsirolimus have been approved by the Food and Drug Administration (FDA) for treating kidney and breast cancers in clinic (Basho et al., 2017; Chan et al., 2005; Vinayak & Carlson, 2013). Nonetheless, due to the insufficient inhibition of mTORC1 substrates (Faes, Demartines, & Dormond, 2017; Thoreen & Sabatini, 2009), the efficacy of rapalogs as anti-tumor drugs is limited. Moreover, a durable inhibition of S6K1 triggers increased Akt activation due to the PI3K-Akt feedback loop (Faes et al., 2017) which accelerates the activation of oncogenic signaling pathways (Chaturvedi, Gao, Cohen, Taunton, & Patel, 2009; Rodrik-Outmezguine et al., 2011). The second-generation inhibitors bind the ATP-active site of mTOR (see **Figure 4**) and therefore inhibit both mTORC1 and mTORC2 (Benjamin, Colombi, Moroni, & Hall, 2011). In pre-clinical models, the anti-tumor efficacy of ATP-competitive TOR inhibitors are generally superior to rapalogs due to the

complete inhibition of mTOR and sustained inhibition of 4E-BP1 phosphorylation. However, complete inhibition of mTOR by the second-generation inhibitors can cause severe side effects and leads to reduced tolerance in the clinic because of kinase similarities between mTOR and other kinases such as PI3K (Benjamin et al., 2011). For example, MLN0128 showed limited clinical efficacy in a clinical Phase II trial due to dose reduction secondary to toxicity (Graham et al., 2018).

Therefore, industry and academia strive to develop better mTOR inhibitors, aiming to maintain mTORC1 selectivity with high potency. Recently, the third-generation mTOR inhibitors emerged, the first one called RapaLink-1 (Rodrik-Outmezguine et al., 2016), which combines allosteric effects and targeting of the active site in a bivalent way. Although this inhibitor shows promising results (Fan et al., 2017), its selectivity is modest which motivated development of inhibitors with increased selectivity for mTORC1 over mTORC2. More details are provided under **Paper I**.

1.4.2 Targeting the translation machinery

There are already a number of therapeutic agents that efficiently target components (e.g. eIF4E, eIF4A) of the translation machinery (Chu & Pelletier, 2018; Hsieh & Ruggero, 2010; Pal, Safari, Jovanovic, Bates, & Deng, 2019; Sharp & Fan, 2021). Moreover, targeting mRNA translation holds promise as an anti-tumor strategy to overcome insensitivity or resistance arising from genetic heterogeneity (Bhat et al., 2015a).

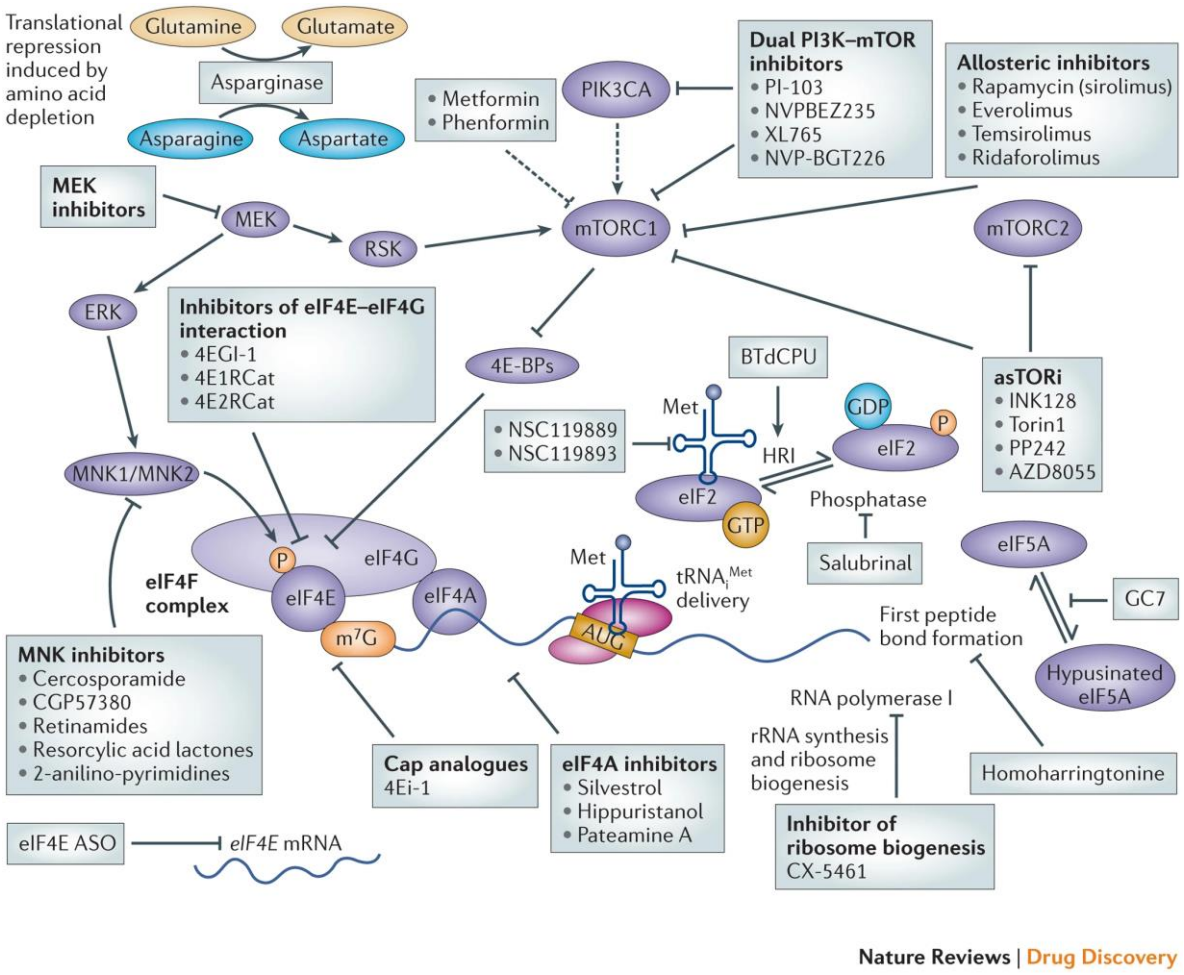

Figure 6: Schematic representation of therapeutic targeting the translation machinery in cancer. Important drug targets of the translation machinery and compounds that target them are shown.

Reprinted with permission from Springer Nature, Nature Reviews Drug Discovery. Bhat, M., Robichaud, N., Hulea, L. et al. Targeting the translation machinery in cancer. Nat Rev Drug Discov 14, 261–278 (2015). https://doi.org/10.1038/nrd4505

1.4.2.1 Direct targeting of the eIF4F complex

As described in section 1.2.1, eIF4E is critical for the assembly of eIF4F and its function. For targeting the eIF4F complex, antisense oligonucleotides (ASOs) against eIF4E have been developed. In mouse models, ASO strategies to suppress eIF4E have been well-tolerated and shown anti-tumor potential (DeFatta, Nathan, & De Benedetti, 2000; Lin et al., 2012; Rinker‐Schaeffer, Graff, De Benedetti, Zimmer, & Rhoads, 1993). Another approach to interrupt eIF4F is via inhibiting the eIF4E-cap interaction. This is supported by the conclusion that eIF4E mutants which are defective in cap binding are not tumorigenic (Wendel et al., 2007). Thus, cap analogs (e.g. 4Ei-1) which inhibit cap-dependent translation have been designed (Ghosh et al., 2009). Overexpression of eIF4G (the scaffolding protein in eIF4F) and eIF4A (the RNA helicase in eIF4F) is also detected in several malignancies and is associated with disease progression (Shanhui Liang et al., 2014; Silvera et al., 2009). eIF4G binds not only eIF4E, but also mRNAs to stabilize the cap-eIF4E association (Moerke et al., 2007). Several agents including 4EGI-1, 4E1RCat and 4E2RCat were designed to inhibit the eIF4E-eIF4G interaction

(R. Cencic et al., 2011; Moerke et al., 2007) and showed promises in pre-clinical models (L. Chen et al., 2012; Mader, Lee, Pause, & Sonenberg, 1995). Hippuristanol, pateamine A, and silvestrol are three eIF4A inhibitors which inhibit translation and show pre-clinical efficacy in both in vitro and in vivo models (M.-E. Bordeleau et al., 2008; R. Cencic et al., 2013; Regina Cencic et al., 2009; Kuznetsov et al., 2009; Low et al., 2014; Malina, Mills, & Pelletier, 2012; Tsumuraya et al., 2011).

1.4.2.2 Interrupt ternary complex formation

In section 1.2.1, the role of TC in translation initiation is described. Although the biochemistry of TC formation and activity is well studied, its role in cancer biology and exploitation as an anti-tumor target is not as well understood as for the eIF4F. Increased phosphorylation of eIF2α, which is the substrate of stress-sensing kinases as noted above, can induce cell apoptosis (Donzé et al., 2004). Therefore, approaches aiming to increase eIF2α phosphorylation have been assessed for cancer treatment. These approaches include the development of salubrinal. (More details in **Table 1, Figure 6**). However, the toxicity of these compounds has been noted, which limited further use.

Table 1: Therapeutic agents targeting translation machinery in cancer (Bhat et al., 2015b; Chu & Pelletier, 2018; Sharp & Fan, 2021).

Target	**Mechanism**	**Name**	**Category**	**References**
eIF4E–Cap Interaction	Inhibit eIF4E cap-binding activity	4Ei-1	An N7-benzyl GMP tryptamine phosphoramidate pronucelotide	(Wagner, Iyer, & McIntee, 2000)
eIF4E:eIF4G Interaction	4EGI-1 binds to eIF4E at a location removed from the eIF4G-binding site	4EGI-1	Compound	(Moerke et al., 2007; Papadopoulos et al., 2014)
	Prevent the association of both eIF4G and 4EBP1 with eIF4E	4E1RCat 4E2RCat		(Regina Cencic et al., 2011)
eIF4A	Bind eIF4A	Pateamine A (PatA)	A naturally occurring metabolite isolated from the marine sponge Mycale hentscheli;	(Northcote, Blunt, & Munro, 1991)
	Prevent eIF4A from interacting with RNA without affecting ATP binding	Hippuristanol	A polyoxygenated steroid first isolated from the gorgonian Isis hippuris;	(M. E. Bordeleau et al., 2006; Higa, Tanaka, & Tachibana, 1981; Higa, Tanaka, Tsukitani, & Kikuchi, 1981)
	Interact directly with eIF4A	Rocaglates; Synthetic rocaglates: Silvestrol; Zotatifin (eFT226)	Rocaglates originated from plants of the Aglaia genus.	(Chambers et al., 2013)
eIF4G	Bind to eIF4G1	BI-69A11 SBI0640756 (SBI-756)	AKT inhibitors	(Gaitonde et al., 2009) (Feng et al., 2015)
eIF2	Blocking dephosphorylation of phospho-eIF2α	Salubrinal	A more potent and soluble derivative (Sal003)	(Robert et al., 2006)
	Prevent the binding of Met-$tRNA_i^{Met}$ to eIF2	NSC119889 NSC119893	Fluorescein derivatives	(Robert et al., 2006)
eIF2B	Cause dimerization of eIF2B	ISRIB (ISRIB-A1; ISRIB-A17)		(Sidrauski et al., 2013) (Sidrauski et al., 2015) (Hearn et al., 2016)

1.5 METHODS TO STUDY TRANSLATION (LIMITED TO THIS THESIS)

To monitor changes of translation efficiency (see definition in section 1.3.1), levels of translated mRNAs (i.e. mRNAs associated with ribosomes) are compared to total mRNA levels in the cytosol.

As translation initiation is rate-limiting under most conditions, the number of translation initiation events per mRNA and unit time approximates the protein synthesis output, and can be assessed by the number of ribosomes associated with the mRNA (see section 1.3.1). Therefore, mRNAs can be separated by velocity sedimentation when loaded on a linear sucrose gradient (5%-50%), followed by ultracentrifugation (a typical polysome tracing plot is in **Figure 7)**.

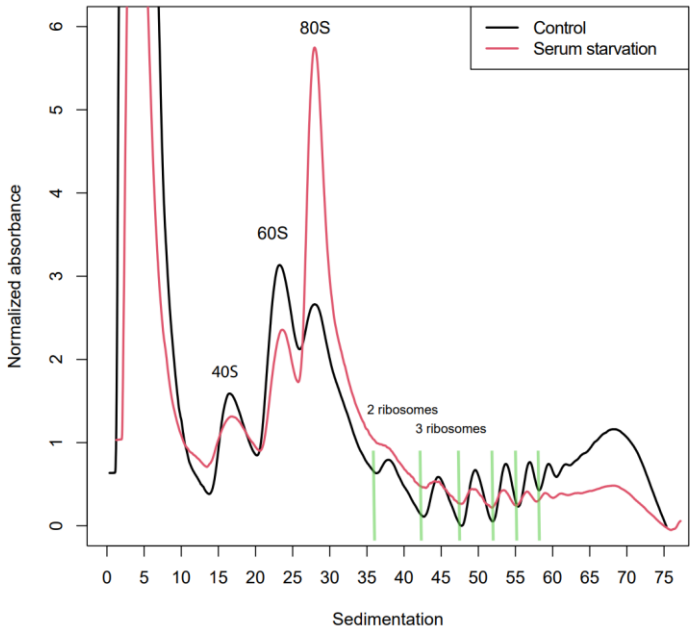

Figure 7: A typical polysome tracing plot to see the shift of transcripts from being highly translated to being suppressed upon a starvation condition. 40S and 60S are subunits of one ribosome. 80S represents one ribosome formation. Green lines define different fractions which contain mRNAs being bond with different number of ribosomes, e.g. two ribosomes, three ribosome, etc. Under starvation, the polysome fractions will decrease compared with the non-starved condition, whereas the 80S part will be increased, meaning the accumulation of monosome, which is a sign of translation efficiency decrease.

1.5.1 Studies of translation for single mRNA species

If the purpose is to detect changes in translation efficiency of specific mRNAs, mRNA from each fraction in **Figure 7** can be collected for downstream quantification using e.g. Northern blotting or reverse transcription quantitative polymerase chain reaction (RT-qPCR). This could reveal a shift in the polysome-association for specific mRNAs across conditions indicating a change in their translation efficiency, i.e. a change in the number of associated ribosomes. Under stress conditions, most RNA species would shift from a heavy polysomal fraction to a light polysomal fraction, or even sub-polysomal fractions. For example, housekeeping genes (e.g. β-actin) are not affected to a similar extent as TOP mRNAs which drastically shift from heavy polysomal fractions to sub-polysomal fractions upon serum deprivation (Geyer,

Meyuhas, Perry, & Johnson, 1982). This dramatic decrease of polysome-association can be detected via protein synthesis output without altered mRNA level. Hence, to monitor how an RNA feature affects translation, like TOP motif, in **Paper II**, we developed a reporter with the RPL23-TOP motif, which could reflect translation changes under different conditions (more details will be discussed below).

1.5.2 Transcriptome-wide studies of mRNA translation

To study translation at a transcriptome-wide scale **polysome profiling** can be used. Polysome-profiling uses RNA-sequencing (RNA-seq) to quantify mRNAs associated with >3 ribosomes, which are thought to be translated efficiently (Gandin et al., 2014). In parallel, cytosolic mRNAs are also collected. In this way, the final RNA-seq data analysis provides cytosolic mRNA-adjusted changes in polysome-associated mRNA levels downstream of altered translation efficiency. This method was recently optimized to sedimentate efficiently translated mRNAs on a sucrose cushion, which enabled transcriptome-wide studies of mRNA translation in small samples and dramatically reduced the experiment time (Shuo Liang et al., 2018).

To enable analysis, analytical and computational approaches have also been developed (Oertlin et al., 2019). Anota2seq is based on anota, which was developed for polysome-profiling data quantified using DNA-microarrays (Larsson, Sonenberg, & Nadon, 2010, 2011). Anota2seq analysis determines alteration in translation efficiency using a linear model. Analysis using Anota2seq categorizes mRNAs into different gene expression modes (**Figure 8**).

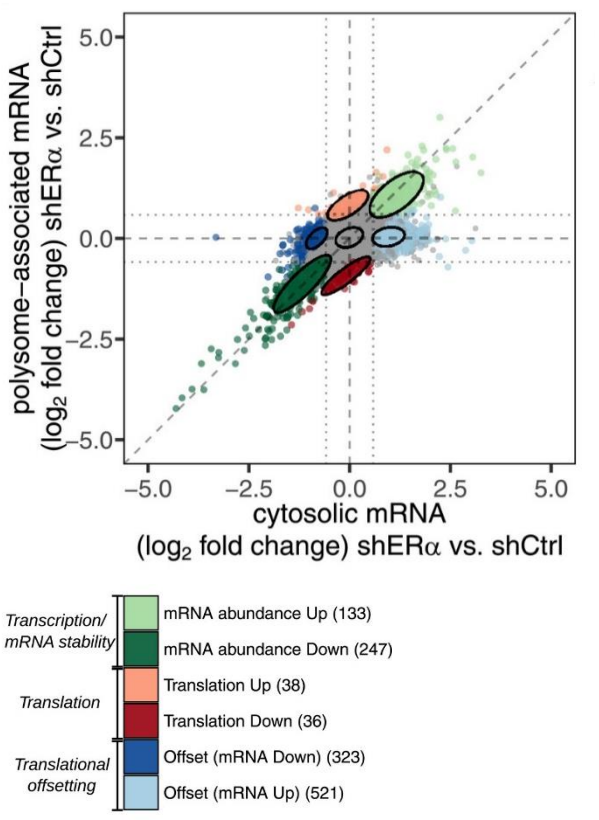

Figure 8: One representative scatter plot of polysome-associated mRNA vs. cytosolic mRNA log2 fold changes.

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Ribosome profiling quantifies ribosome protected fragments and analyses translation by comparing changes in such fragments which cannot be explained by alterations in total cytosolic mRNA (Ingolia, Ghaemmaghami, Newman, & Weissman, 2009; McGlincy & Ingolia, 2017). However, as the RNase digestion only retains the ribosome-protected fragments (RPFs), the information about the number of ribosome association with mRNAs is lost. Nonetheless, it can provide information about the location of ribosome occupancy on mRNAs which enables studies aiming to find regulatory features of mRNA translation (Aeschimann, Xiong, Arnold, Dieterich, & Großhans, 2015).

Moreover, the development of RNA-seq techniques also facilitates transcriptome-wide studies of mRNA translation. For instance, Smart-seq2 library preparation allows transcriptome analysis from single cells, which needs a very low amount of input RNA(Picelli et al., 2014). Recently, nano-cap analysis of gene expression (nanoCAGE) method specifically sequences the 5'UTR of transcripts, which can provide transcription start site (TSS) profiling of translated transcripts in combing with polysome profiling (Gandin et al., 2016). At the individual mRNA level, live cell imaging methods e.g. TRICK, NCT/SINAPS (Halstead et al., 2015; Morisaki et al., 2016; C. Wang, Han, Zhou, & Zhuang, 2016; Wu, Eliscovich, Yoon, & Singer, 2016; Yan, Hoek, Vale, & Tanenbaum, 2016) have also been developed to allow monitor live single-molecule translation in a dynamic fashion.

In this thesis, polysome profiling is used to reveal translational control under different contexts, i.e. effect of selective mTORC1 inhibitors on translatome (**Paper I**), translational control associated with *VHL* status in response to rapamycin (**Paper III**), and translational control of immune cell phenotype (**Paper IV**). In **Paper II**, translation of one subset of mRNAs, i.e. TOP mRNAs is explored with a developed reporter for finding modulators.

2 AIMS OF THIS THESIS

Overall, this thesis aims to study mTOR-dependent regulation of mRNA translation in cancer.

In **study I**, we aimed to examine the effects of newly developed mTORC1-selective bi-steric inhibitors on translatome.

In **study II**, we aimed to develop a luciferase reporter for TOP-mRNAs, whose translation is very mTOR-sensitive to enable the identification of additional factors and pathways modulating such translation either dependent or independent of mTOR.

In **study III**, we aimed to characterize the VHL-associated effects on mRNA translation in response to mTOR inhibition.

In **study IV**, we aimed to unravel whether mRNA translation contributes to TAM phenotypes.

3 RESULTS AND DISCUSSION

3.1 STUDY I--SELECTIVE INHIBITORS OF MTORC1 ACTIVATE 4EBP1 AND SUPPRESS TUMOR GROWTH

PI3K-mTOR pathway affects a wide range of physiological functions and cellular activities. Dysregulation of this pathway due to genomic alterations plays an essential role in carcinogenesis and tumor progression (Chalhoub & Baker, 2009; Vivanco & Sawyers, 2002), and is the second frequently altered pathway after the p53 signaling pathway (Klempner, Myers, & Cantley, 2013). Thus, potent targeting of PI3K-mTOR has been an important therapeutic strategy in cancer (Janes & Fruman, 2010; Thoreen et al., 2009). The first-generation mTOR inhibitors, rapalogs, have been applied in monotherapy or combination therapies (Benjamin et al., 2011; Gonzalez-Angulo et al., 2013). The mechanism whereby rapalogs inhibit mTOR is through the allosteric binding with the FKBP-rapamycin binding (FRB) domain of mTOR (see **Figure 4**). Structural distinctions of components between mTORC1 and mTORC2 (see **Figure 4**) offer the basis for rapalogs with higher selectivity of mTORC1 over mTORC2. However, such inhibition (Choo et al., 2008; Sarbassov et al., 2006) is limited due to insufficient targeting of mTORC1 downstream substrates, such as 4EBP1 and S6K (Bissler et al., 2008; Buti, Leonetti, Dallatomasina, & Bersanelli, 2016; Vinayak & Carlson, 2013). The second-generation mTOR inhibitors are designed to compete with ATP in the catalytic site of mTOR, thus suppressing both mTORC1 and mTORC2 (Zhou & Huang, 2012). Yet, the clinical benefits remain unsatisfying (Graham et al., 2018; Powles et al., 2016). Recently, a third-generation inhibitor, RapaLink-1 (Rodrik-Outmezguine et al., 2016), which exploits the unique juxtaposition to covalently link two drug-binding pockets, was reported to overcome the resistance to existing first- and second-generation inhibitors. Of note, RapaLink-1 inhibits mTOR more potently, but still low selectivity for mTORC1 (only three- to four-fold) (Fan et al., 2017; Rodrik-Outmezguine et al., 2016).

With this prototype, RapaLink-1, we modified the essential core moieties to produce compounds with varying mTORC1/2 selectivity. From the biochemical and pharmacological data, the selectivity of these compounds was identical with the affinities of allosteric (mTORC1) and orthosteric (mTORC1 and mTORC2) components for their respective binding sites. Among those, three mTORC1-selective bi-steric inhibitors (BiS-13x, BiS-31x, and BiS-35x) were further evaluated for their biological characterization compared with pan-mTOR active-site (MLN0128) and bi-steric (BiS-NS) inhibitors or rapamycin.

The basis of selectivity of mTORC1 over mTORC2 relied on the formation of FKBP12-mTOR-inhibitor ternary complex. To verify if our modular approach affects the spectrum of kinases, a mass spectrometry-based kinome screen (Patricelli et al., 2007) was conducted and analysis of kinase interactions found interaction occurring solely with mTOR in BiS-NS and BiS-31x. Even though BiS-13x and BiS-35x were observed to interact with certain kinases, the extent was much lower than that in pan-mTOR active-site inhibitors (MLN0128 and PP242). These results indicated mTORC1-selective bi-steric inhibitors would not significantly inhibit other kinases and have less off-target effects.

The anti-tumor effect in pre-clinical in-vitro models showed that BiS-13NS and BiS-13x have a higher potency than BiS-31x and BiS-35x, as well as MLN0128, which was consistent with the induced caspase activity. BiS-35x, while showing the least potency among bi-steric selective inhibitors, still induced higher caspase activity at a concentration (100 nM) than rapamycin (1 uM). Importantly, from the in vitro results, bi-steric mTORC1-selective inhibitors did not induce receptor tyrosine kinase expression, which was due to induced AKT activition. This might be the molecular basis for the reduced glucose intolerance of selective inhibitors observed in vivo (more details below).

To verify the 4EBP1 dependency of mTORC1-selective bi-steric inhibitors in suppressing tumor growth, we made the variant, 4EBP1-A, which was a doxycycline-inducible 4EBP1 mutant protein. It showed 4EBP1-A was insensitive to inhibition of mTOR, and maintained a constitutively active form of 4EBP1 upon mTOR kinase inhibition. In-vitro results showed that effective inhibition of 4EBP1 phosphorylation was sufficient to suppress tumor proliferation. Further, we also used CRISPR/Cas9 editing and shRNA techniques to delete or knockdown 4EBP1 from the in vitro cancer cell line to examine the necessity of active 4EBP1 in such inhibition of tumor growth. As expected, effects of these inhibitors on eIF4F complex formation and eIF4F-sensitive translation were attenuated, yet only a modest reduction of anti-proliferation effect upon pan-mTOR inhibitor (RapaLink-1) and mTORC1-selective bi-steric inhibitors (BiS-13x and BiS-35x) was observed. A similar pattern was also observed in 4EBP1/2 DKO MEFs upon MLN0128, and BiS-13x, BiS-35x compared to 4EBP1/2 wild-type MEFs. Taken together, 4EBP1 activation is necessary for maximal inhibition of tumor proliferation by both pan-mTOR inhibitors and mTORC1-selective bi-steric inhibitors, but not necessary for a significant anti-proliferation activity.

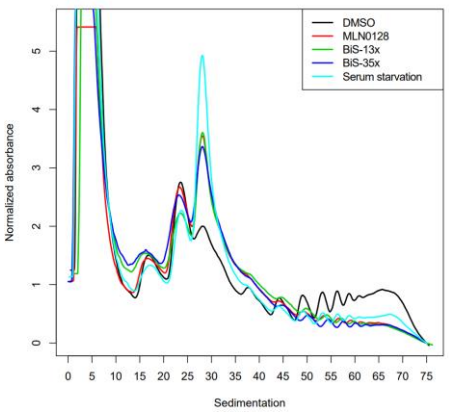

Figure 9: Representative polysome tracings of inhibition with indicated conditions.

Accordingly, transcriptome-wide comparisons of translation efficiencies were obtained using polysome profiling (section 1.5.2, **Figure 9**) between active-site inhibitor MLN0128 and two mTORC1-selective bi-steric inhibitors, BiS-13x and BiS-35x. Notably, the effects on mRNA translation and total mRNA levels were identical for BiS-13x, BiS-35x as compared to MLN0128. This therefore supports that mTORC1-selective bi-steric inhibitors show similar potency as an active-site inhibitor for mTORC1 inhibition, while having higher selectivity for mTORC1 over mTORC2. Moreover, these studies also indicate that mTORC1, but not mTORC2 is responsible for the translational reprogramming downstream of mTOR inhibition.

Further, pan-mTOR inhibitors (MLN0128 and BiS-NS) and mTORC1-selective inhibitors (BiS-13x and BiS-31x) were evaluated in an MCF7 subcutaneous xenograft model using an administration schedule previously used for RapaLink-1 (Rodrik-Outmezguine et al., 2016). Apart from MLN0128, the inhibitors all showed sustained inhibition of phosphorylation of 4EBP1 in plasma. Moreover, BiS-13x and BiS-31x induced increased phosphorylation of AKT on S473 site at 4 hours of administration (sustained for at least 72 hours) which was much earlier than the pan-mTOR inhibitor, BiS-NS. Hence, the in vivo results for mTORC1-selective bi-steric inhibitors were identical with in-vitro experiments, with selective inhibition of p4EBP1 while inducing pAKT S473. And the anti-tumor effect in vivo of BiS-13x and BiS-31x was also comparable with MLN0128 and correlated with inhibition of p4EBP1, but not pAKT S473. Meanwhile, mice body weights were also recorded as a measure of drug toxicity. It turned out compounds with higher selectivity for mTORC1 showed better tolerance, with maximum weight loss by BiS-13x and BiS-31x of 10% and 5%, respectively. A similar observation was also shown in the xenograft model with another breast cancer cell line.

As discussed above, AKT inhibition by pan-mTOR inhibitors, particularly, inhibition of AKT S473 phosphorylation may be related with glucose intolerance upon PI3K inhibition (Hagiwara et al., 2012). When mice were administered to the candidate mTORC1-selective bi-steric inhibitors and pan-mTOR inhibitors, glucose levels in the blood were traced with a two-day glucose tolerance test (GTT) to monitor the immediate and delayed effects. Significantly, MLN0128 delayed the return of glucose levels to baseline after both the first and second glucose challenge, and the modified-version of RapaLink-1, BiS-NS also had an effect on glucose recovery at the delayed phase. In contrast, in mice administered with mTORC1-selective bi-steric inhibitors, BiS-13x and BiS-31x, blood glucose levels returned to baseline within one or two hours after the challenge, showing better glucose tolerance, due to the relief of mTORC2-dependent inhibition of AKT. However, the association needs further experimental validation.

In summary, mTORC1-selective bi-steric inhibitors developed via modular approach are, to our knowledge, the first compounds to achieve significant inhibition selectivity of mTORC1 over mTORC2, whereby suppression of 4EBP1 was sufficient for anti-tumor activity in pre-clinical models and caused less glucose intolerance. As dissected using polysome profiling for comparisons of different mTOR inhibitors, targeting of mTORC1 provided a translational reprogramming associated with the antitumorigenic effects of mTOR inhibition.

3.2 STUDY II--IDENTIFICATION OF KINASES MODULATING TRANSLATION OF MRNAS WITH TERMINAL OLIGO-PYRIMIDINE (TOP) MOTIFS

Regulation of gene expression via modulation of translation efficiencies is an important means to shape the protein repertoire during adaption to internal or environmental alterations (see section 1.3). Terminal oligopyrimidine (TOP) mRNA is a group of transcripts with a specific sequence feature in their 5'UTR, i.e. a stretch of uninterrupted pyrimidines. Such sequence

feature confers the sensitivity of TOP mRNA to regulation of the mTOR signaling pathway. However, other regulators acting dependently or independently of mTOR pathway are incompletely characterized. LARP1, as discussed above, acts as a negative regulator of TOP mRNA translation downstream of mTORC1. Yet, its depletion could not completely re-depress the inhibition on TOP mRNA translation by mTOR inhibitors such as rapamycin or torin1. This suggests that other regulators may also play a role in such regulation. Therefore, a precise and sensitive reporter for TOP mRNA translation in combination with a kinome-wide siRNA-based screening strategy was implemented for the exploration of candidate regulators.

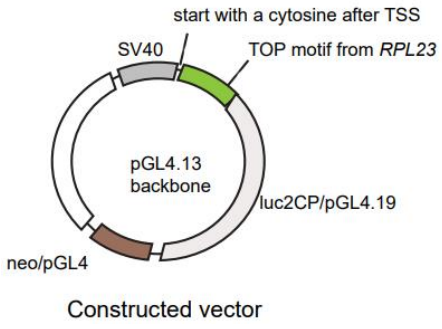

Figure 10: A map of the pGL4.13[RPL23-luc2CP/SV40/Neo] reporter plasmid.

To generate a reporter for TOP mRNA translation, we generated the pGL4.13[RPL23-luc2CP/SV40/Neo] vector (**Figure 10**). Precisely, the 5'UTR from one classical TOP mRNA, *RPL23*, was positioned at the transcript start site (TSS) of the SV40 promoter. This was further verified using the 5' rapid amplification of cDNA ends (RACE) approach (**Figure 11**). In fact, this step was challenging as the TSS needed to be precise to include a cytosine at the first position whereas this is usually not required when constructing expression vectors. Moreover, a stably transfected cell line was also established and the response of the reporter under alterations in mTOR activity was evaluated using insulin stimulation in the presence or absence of an mTOR inhibitor torin1. This revealed that the reporter vector could detect changes in the protein levels independent of mRNA abundance (**Figure 12**), a hallmark of TOP mRNAs following modulation of mTOR activity.

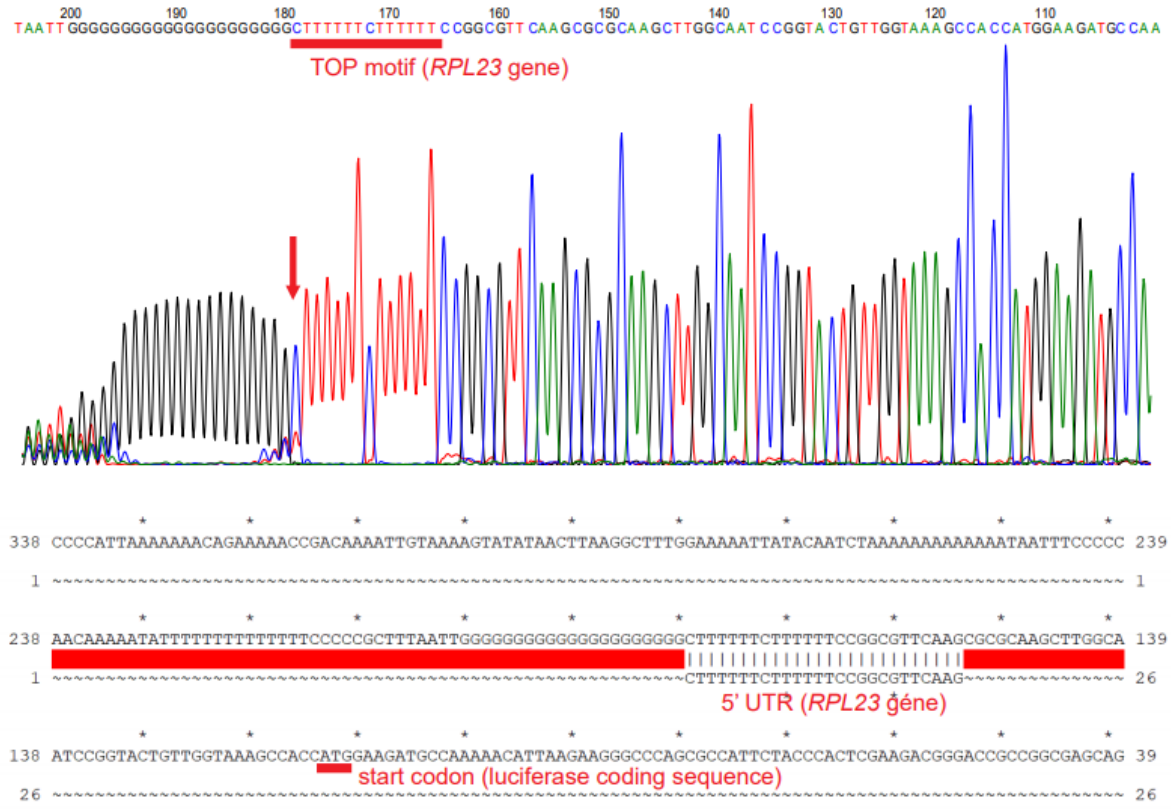

Figure 11: 5'RACE-based validation of expression of luc2CP mRNA with the TOP motif from RPL23 at the +1 position. The position of the TOP motif immediately follows the poly-G stretch which marks the TSS in 5'RACE (upper panel). The entire RPL23 5'UTR is indicated relative to the start codon for the luc2CP gene (lower panel).

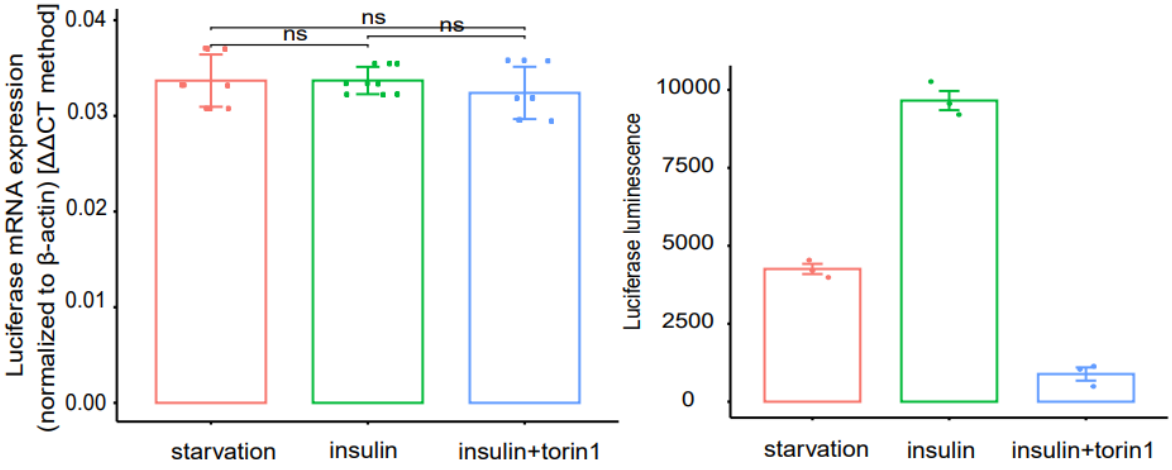

Figure 12: (left) luc2CP mRNA levels were measured under different conditions and normalized to β-actin (mean and standard deviations from 3 independent experiments are shown) (right) Luciferase activity following expression of the RPL23-luc2CP reporter were quantified under the same conditions as in (left). Mean and standard deviations from 3 independent experiments are shown.

Next, we applied a cell-based assay for screening of possible regulators modulating translation of TOP mRNA. Consistent with current knowledge regarding the role of LARP1 in regulation of TOP mRNA (Fonseca et al., 2015b; Lahr et al., n.d.; Philippe et al., 2018), LARP1 was identified as a negative regulator in our screen. As expected, the silencing of Raptor showed a significant decrease in TOP mRNA translation. Through this approach, multiple kinases were identified as candidate regulators of TOP mRNA translation. Herein, we focused on a few previously suggested or uncharacterized regulators of TOP mRNA translation. Following reduced levels of specific amino acids such as cysteine/cystine, GCN2 is activated and catalyzes eIF2α phosphorylation, thereby inducing the expression of genes such as ATF4. Recently, it was reported that GCN2-eIF2α axis controls TOP mRNAs translation (Li et al., 2018a). Interestingly, in our assessment, depletion of GCN2 by siRNA also induced increased TOP mRNA translation under serum starvation as well as mTOR inhibition. Moreover, such regulation was also not dependent on LARP1, according to the dual depletion of GCN2 and LARP1, whereas increased translation by siGCN2 was abolished by dual depletion of GCN2 and Raptor. Although these studies suggest GCN2 as part of a parallel pathway controlling TOP mRNA translation, the detailed mechanisms remain to be elucidated. Similarly, we also identified MAPK13, a member of MAP kinase family, which also appeared to be a negative regulator of TOP mRNA translation.

3.3 STUDY III--IN VITRO CHARACTERIZATION OF VHL DEPENDENT MRNA TRANSLATION IN RENAL CELL CARCINOMA

mRNA translation is regulated under low oxygen level (see section 1.3.3.2). In fact, most solid tumors experience transient or prolonged low oxygen supply during progression due to abnormal angiogenesis. In the specific context of renal cell carcinoma, the most common genetic alteration is loss of function in *VHL*. Being the tumor suppressor, pVHL is responsible for the degradation of HIF, whereas loss of *VHL* can lead to increased HIF expression even under normal oxygen condition. In paper III, we used in vitro RCC4 cell model to compare the effect of *VHL* in combination with polysome profiling to dissect how VHL loss affects mRNA translation. Although rapalogs, everolimus and temsirolimus, have been approved by FDA for a systematic treatment of RCC, whether patients harboring different mutations or other biomarkers respond differently to rapalogs is not well understood. One cohort study (Kwiatkowski et al., 2016), reported that patients with mutations in *mTOR* or its suppressor *TSC1/2* in metastatic RCC patients benefited more from rapalogs. But, among responders, 24 out of 43 were identified without mTOR pathway mutation. To figure out whether *VHL* status also has an impact on response to rapalogs, we treated VHL-proficient and VHL-deficient cell lines with rapamycin, and applied polysome-profiling in conjunction with anota2seq analysis to dissect transcript-selective translation modes.

By comparing VHL-proficient RCC to VHL-deficient RCC, wide-spread VHL-associated modulation of gene expression at the mRNA level as well as the translation level was revealed. In particular, we sought to address whether the rapamycin response depends on VHL status.

As shown in **Figure 13**, although VHL-proficient cells showed numerous alterations in cytosolic mRNA level following 2 h of rapamycin treatment (marked in light blue and dark blue), their polysome-association remained largely unaltered. Such genes therefore show buffering at the level of translation. In contrast, a set of genes (marked in orange and dark red) did not change their cytosolic mRNA abundance but showed altered levels of polysome-associated mRNA. Such transcripts were regulated via changes in translation independent of cytosolic mRNA abundance. Only a small subset of genes in VHL-deficient cells were down-regulated in the polysome-associated mRNA level by rapamycin. Similarly, we assessed 16 h rapamycin treatment in a similar fashion (**Figure 14)**. More genes were being disturbed by rapamycin in VHL-deficient cells. Functionally, genes uniquely sensitive to rapamycin in VHL-proficient cells, encoded proteins which were not significantly annotated to GO terms or KEGG pathways except for the interleukin-1-mediated signaling pathway. Of note, upon prolonged rapamycin treatment, genes which were translationally induced in response to rapamycin only in VHL-proficient cells were significantly enriched in immunity-related terms and important signaling pathways such as neurotrophin signaling pathway. In turn, genes which were translationally suppressed in response to rapamycin only in VHL-deficient cells were enriched in nucleosome- or chromatin-assembly related terms. In summary, genes in VHL-proficient cells were not present in VHL-deficient cells in response to rapamycin, vice versa, were identified, and enriched with distinct functionalities. These studies therefore revealed ample regulation of translation depending on VHL and rapamycin in RCC.

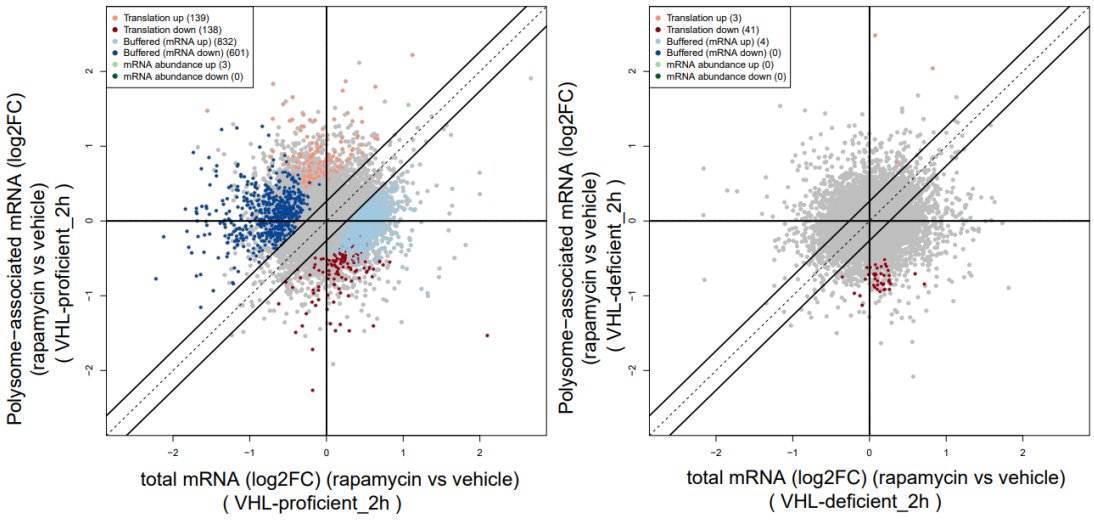

Figure 13: Visualization of rapamycin-sensitive gene expression following 2 h of serum stimulation in presence or absence of rapamycin in VHL proficient (left) and deficient (right) cells.

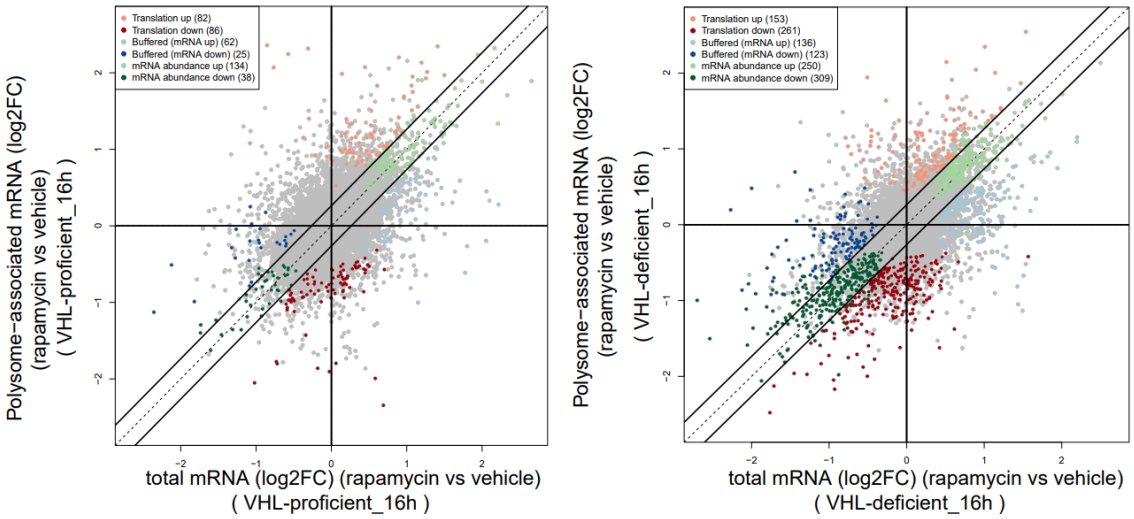

Figure 14: Similar to Figure 13 but assessing rapamycin-sensitive gene expression following 16 h of serum stimulation in presence or absence of rapamycin.

3.4 STUDY IV--MNK2 GOVERNS THE MACROPHAGE ANTIINFLAMMATORY PHENOTYPE

The role of eIF4F formation in regulating cap-dependent translation has been described in section 1.3.2.2. 4EBP1, as the suppressor of the key component eIF4E in the eIF4F complex, is modulated downstream of mTOR signaling pathway. mTOR promotes phosphorylation of 4EBP1, and whereby eIF4E gets released to associate with eIF4G and eIF4A to form the eIF4F complex. Another modulator of eIF4E function is MNK1/2 which is responsible for phosphorylation of eIF4E. In **paper IV**, we observed tumor-weight-associated changes in mRNA translation in tumor-associated macrophages (TAMs). During tumor growth, there is a shift in the TAM population from a predominantly proinflammatory phenotype toward an antiinflammatory phenotype (**Figure 15**). Interestingly, further validation revealed that the MNK2/eIF4E but not the mTOR/4EBP axis controlled expression of transcripts encoding proteins related with the antiinflammatory phenotype of TAM. Accordingly, modulating MNK2/eIF4E via cercosporamide (chemical MNK inhibitors), depletion of MNK2 by lentiviral vectors encoding shRNAs targeting MNK2, or mutation of the MNK1/2 phosphorylation site on eIF4E in ex vivo/in vivo setups, showed almost identically increased activation of $CD8^+$ T cells.

Tumor cells, especially in solid tumors, are always surrounded by tumor microvessels and immune cells in the stroma, constituting the tumor microenvironment (TME). Cross-talk between cancer cells and immune cells plays a decisive role in tumor progression and immune activity (Hinshaw & Shevde, 2019), which underlies the basis of immunotherapy in cancer treatment. Combining with the finding in **paper I** that activation of 4EBP1 is sufficient for maximal inhibition of proliferation by mTORC1-selective bi-steric inhibitors, but is not necessary for significant anti-proliferative activity, it seems eIF4F activity mediated by eIF4E phosphorylation could be another targetable component for achieving improved anti-tumor

activity. Moreover, such inhibition may have a potential role in increasing cytotoxic $CD8^+$ T cell activity. Results from **paper IV** indicated that combined shMNK2 depletion and MNKi inhibition on TAM did not induce higher IFNγ production in $CD8^+$ T cells than either of the two strategies. This suggested a ceiling for MNK2/eIF4E axis-dependent induction of $CD8^+$ T cell activity that existed in TAM. However, we did not look further into how dual targeting of 4EBP1 and phosphorylation of eIF4E affect tumor cell proliferation or immune cell cytotoxic activity. Nonetheless, we believe phosphorylation of eIF4E could partially contribute to the suppressive immune response via TAM functional phenotype shift during tumor growth.

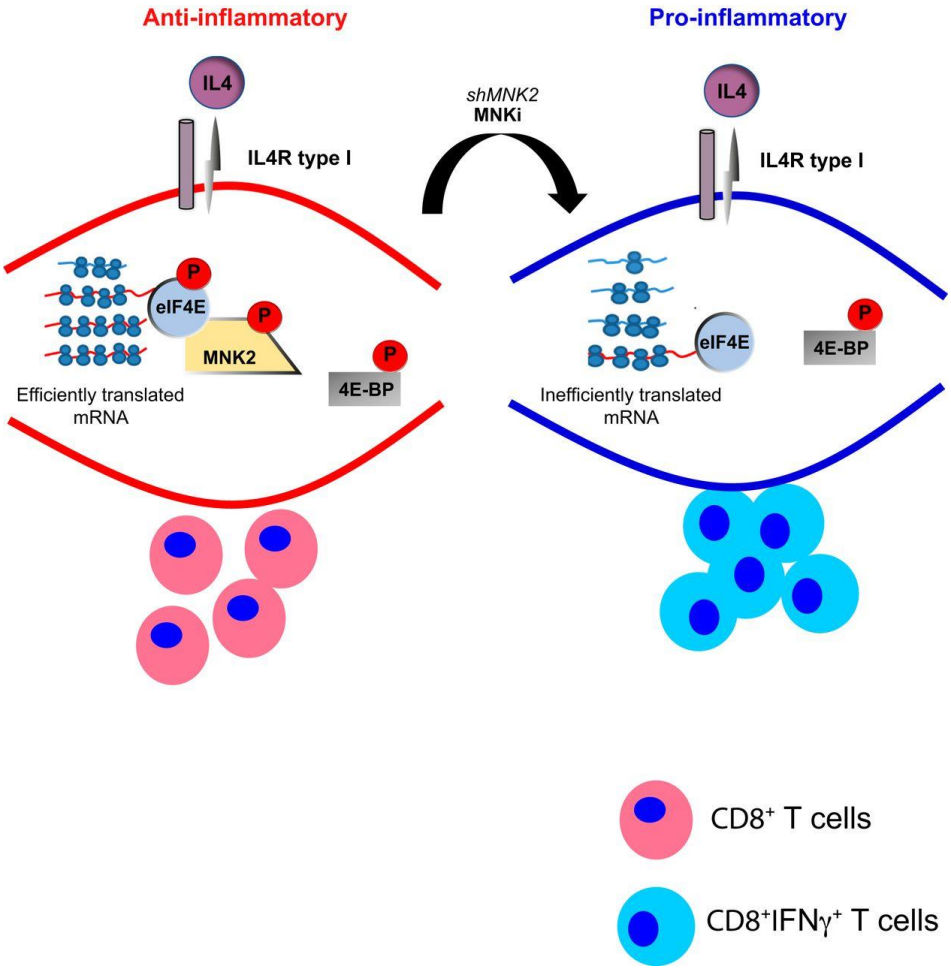

Figure 15: Schematic of MNK2-dependent control of the antiinflammatory Mφs phenotype.

4 CONCLUSION AND FUTURE PERSPECTIVE

Gene expression is orchestrated at multiple levels in eukaryotic cells. This thesis specifically provides insights into the role of mRNA translation, which acts as the distal step in conveying genomic messages to functional protein molecules. One focus is how the translation of transcripts is modulated via the mTOR pathway independently of their mRNA levels. Importantly, this control is preferential for transcripts with distinct structural and/or nucleotide-sequence features. Functionally, control of those transcripts' translation has profound biological consequences by reshaping the proteome towards a malignancy, an altered functional phenotype, or acquired resistance to treatment. Accordingly, elegant interrogation of mRNA translation through mTOR-mediated control or other pathways could open new avenues for cancer treatment.

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Even when I write this part of the thesis now, I can't believe how far I have come during my PhD journey. In the past five years, it was not only diving into science, but also mentally experiencing growing up to be sensible. There were so many kind persons around accompanying me. Without you, it would not have been possible to arrive here. I may not thank everybody who deserves it here, since these pages are far too few for that. Thus, the acknowledgments are limited to those people who made direct contributions towards this thesis.

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